US009260725B2

(12) **United States Patent**
**Trono et al.**

(10) **Patent No.: US 9,260,725 B2**
(45) **Date of Patent: *Feb. 16, 2016**

(54) **METHODS AND COMPOSITIONS RELATING TO IMPROVED LENTIVIRAL VECTOR PRODUCTION SYSTEMS**

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( * ) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **14/525,520**

(22) Filed: **Oct. 28, 2014**

(65) **Prior Publication Data**

US 2015/0203870 A1 Jul. 23, 2015

**Related U.S. Application Data**

(60) Continuation of application No. 12/578,346, filed on Oct. 13, 2009, now Pat. No. 8,900,858, which is a division of application No. 10/209,952, filed on Aug. 1, 2002, now Pat. No. 7,629,153.

(60) Provisional application No. 60/309,569, filed on Aug. 2, 2001.

(51) **Int. Cl.**

| | |
|---|---|
| ***A61K 48/00*** | (2006.01) |
| ***C12N 15/86*** | (2006.01) |
| ***C12N 7/00*** | (2006.01) |

(52) **U.S. Cl.**

CPC ***C12N 15/86*** (2013.01); ***C12N 7/00*** (2013.01); *A61K 48/00* (2013.01);

(Continued)

(58) **Field of Classification Search**

CPC ..... C12N 2740/16043; A61K 48/00

See application file for complete search history.

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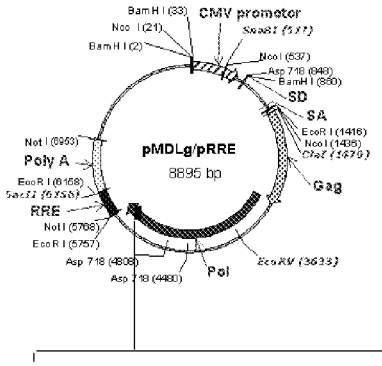
(57) **ABSTRACT**

The present invention provides HIV-derived lentivectors which are multiply modified to create highly safe, efficient, and potent vectors for expressing transgenes for gene therapy. The lentiviral vectors comprise various combinations of an inactive central polypurine tract, a stuffer sequence, which may encode drug susceptibility genes, and a mutated hairpin in the 5' leader sequence that substantially abolishes replication. These elements are provided in conjunction with other features of lentiviral vectors, such as a self-inactivating configuration for biosafety and promoters such as the EF1α promoter as one example. Additional promoters are also described. The vectors can also comprise additional transcription enhancing elements such as the wood chuck hepatitis virus post-transcriptional regulatory element. These vectors therefore provide useful tools for genetic treatments for inherited and acquired disorders, gene-therapies for cancers and other disease, the creation of industrial and experimental production systems utilizing transformed cells, as well as for the study of basic cellular and genetic processes.

**23 Claims, 12 Drawing Sheets**




pMDLg/pRRE 5' AAT TTT AAA AGA AAA GGG GGG 3'

pMDD 5' AAC TTC AAG CGC CGC GGT GGT 3'

(52) **U.S. Cl.**
CPC ..... *C12N 2740/15043* (2013.01); *C12N 2740/16043* (2013.01); *C12N 2740/16052* (2013.01); *C12N 2740/16061* (2013.01); *C12N 2830/38* (2013.01); *C12N 2830/48* (2013.01); *C12N 2830/50* (2013.01); *C12N 2840/203* (2013.01); *C12N 2840/44* (2013.01)

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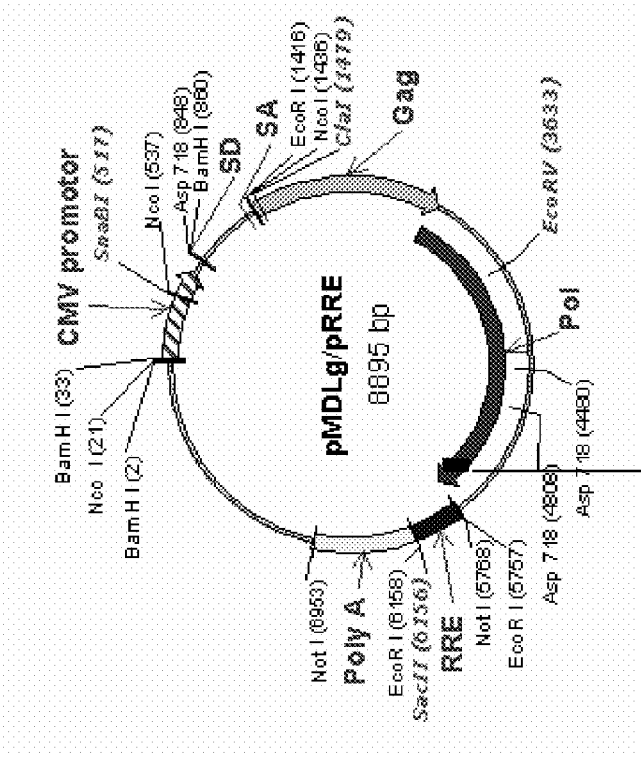
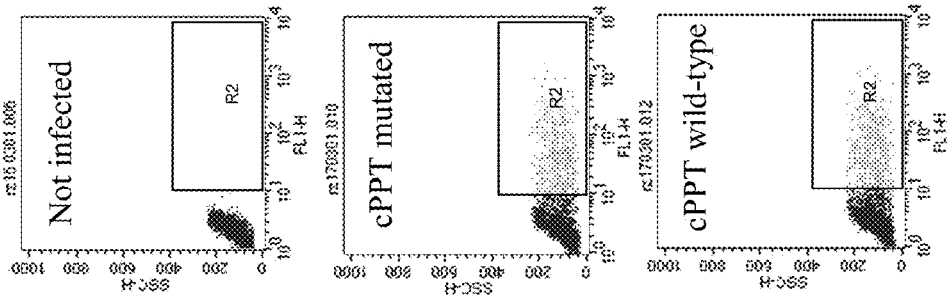



pMDLg/pRRE 5' AAT TTT AAA AGA AAA GGG GGG 3'

pMDD 5' AAC TTC AAG CGC CGC GGT GGT 3'

FIG. 1




| | % GFP positive cells | Mean GFP intensity |
|---|---|---|
| Not infected | 0 % | 0 |
| cPPT mutated | 17.11 % | 93.83 |
| cPPT wild-type | 18.09 % | 100.22 |

FIG. 2

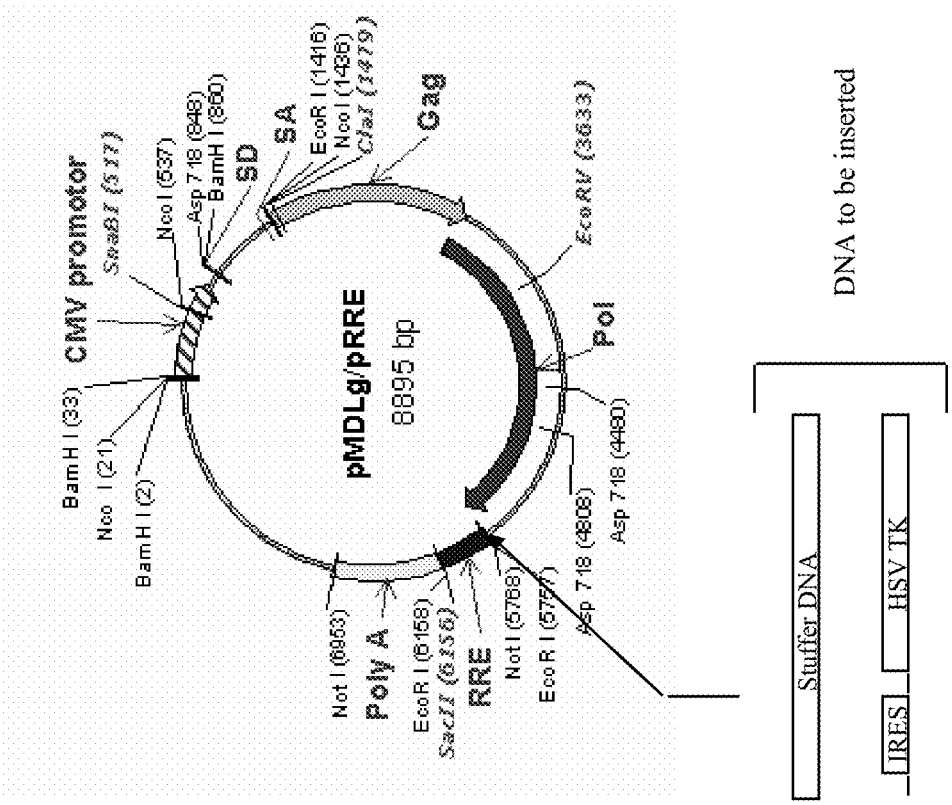

pMDLg/pRRE
8895 bp
CMV promotor
SwaBI (511)
Nco I (537)
Asp 718 (848)
BamH I (860)
SD
SA
EcoR I (1416)
Nco I (1436)
ClaI (1479)
Gag
EcoRV (3633)
Pol
Asp 718 (4480)
Asp 718 (4808)
EcoR I (5757)
Not I (5768)
RRE
EcoR I (6156)
SacII (6158)
Poly A
Not I (6953)
BamH I (33)
Nco I (21)
BamH I (2)
DNA to be inserted
Stuffer DNA
IRES
HSV TK

FIG. 3

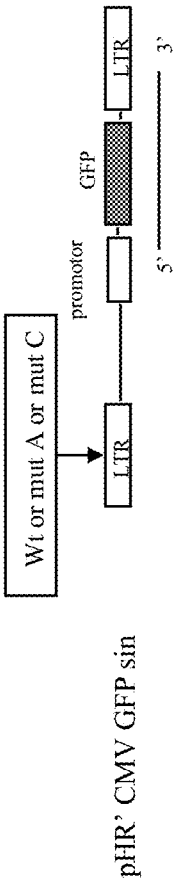
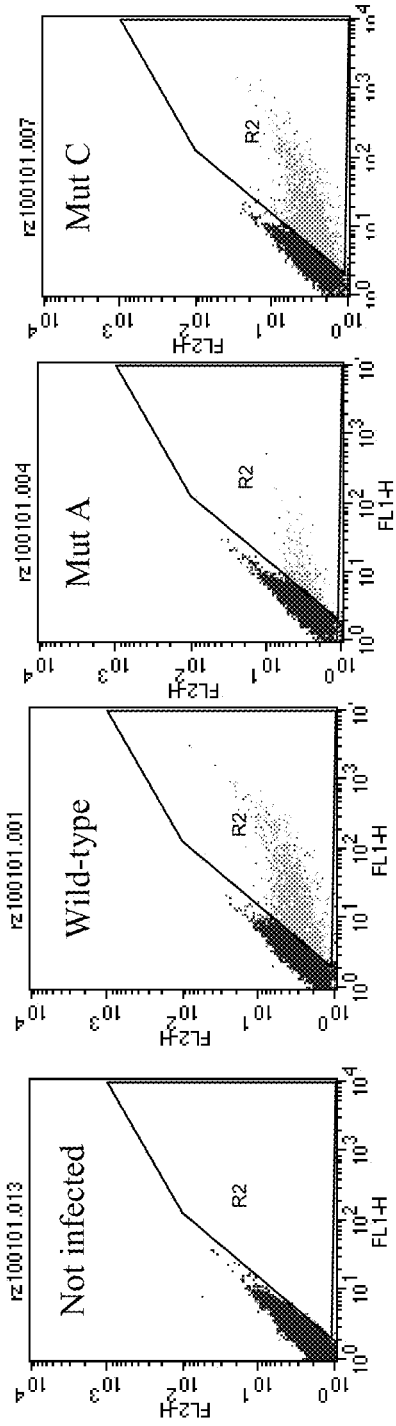
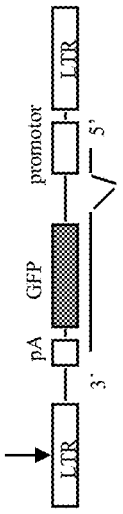



FIG. 4



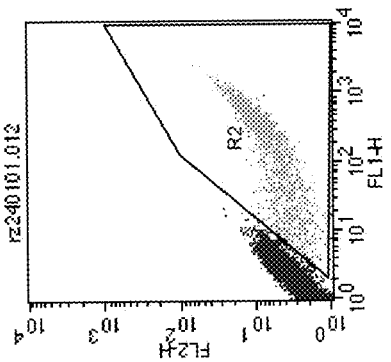

| | Not infected | Wild-type | Mut A | Mut C |
|---|---|---|---|---|
| % GFP positive | 0.06 % | 16.39 % | 1.99 % | 17.38 % |
| M F I | 3.20 | 33.74 | 15.32 | 25.75 |

FIG. 5




pHR' pA GFP I AC

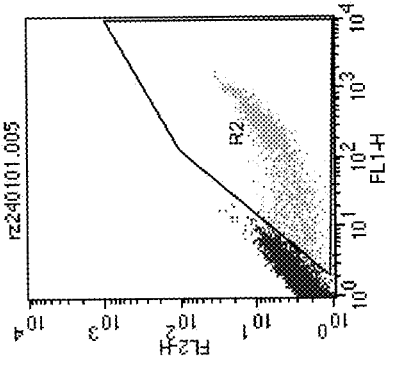
FIG. 6




Wild-type

% GFP positive 22.25%

Mean fluorescence intensity 274.3




Mut A

% GFP positive 19.72%

Mean fluorescence intensity 215.77

FIG. 7

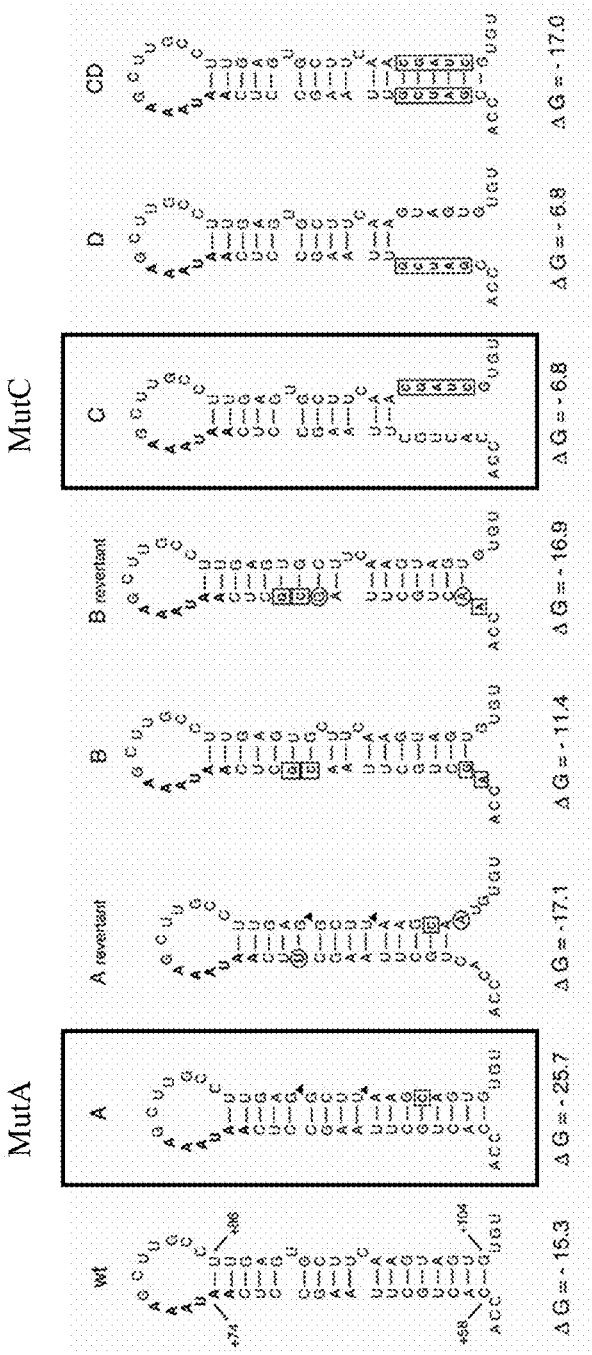

MutA
MutC
wt
A
A revertant
B
B revertant
C
D
CD
ΔG = -15.3
ΔG = -25.7
ΔG = -17.1
ΔG = -11.4
ΔG = -16.9
ΔG = -6.8
ΔG = -6.8
ΔG = -17.0

FIG. 8

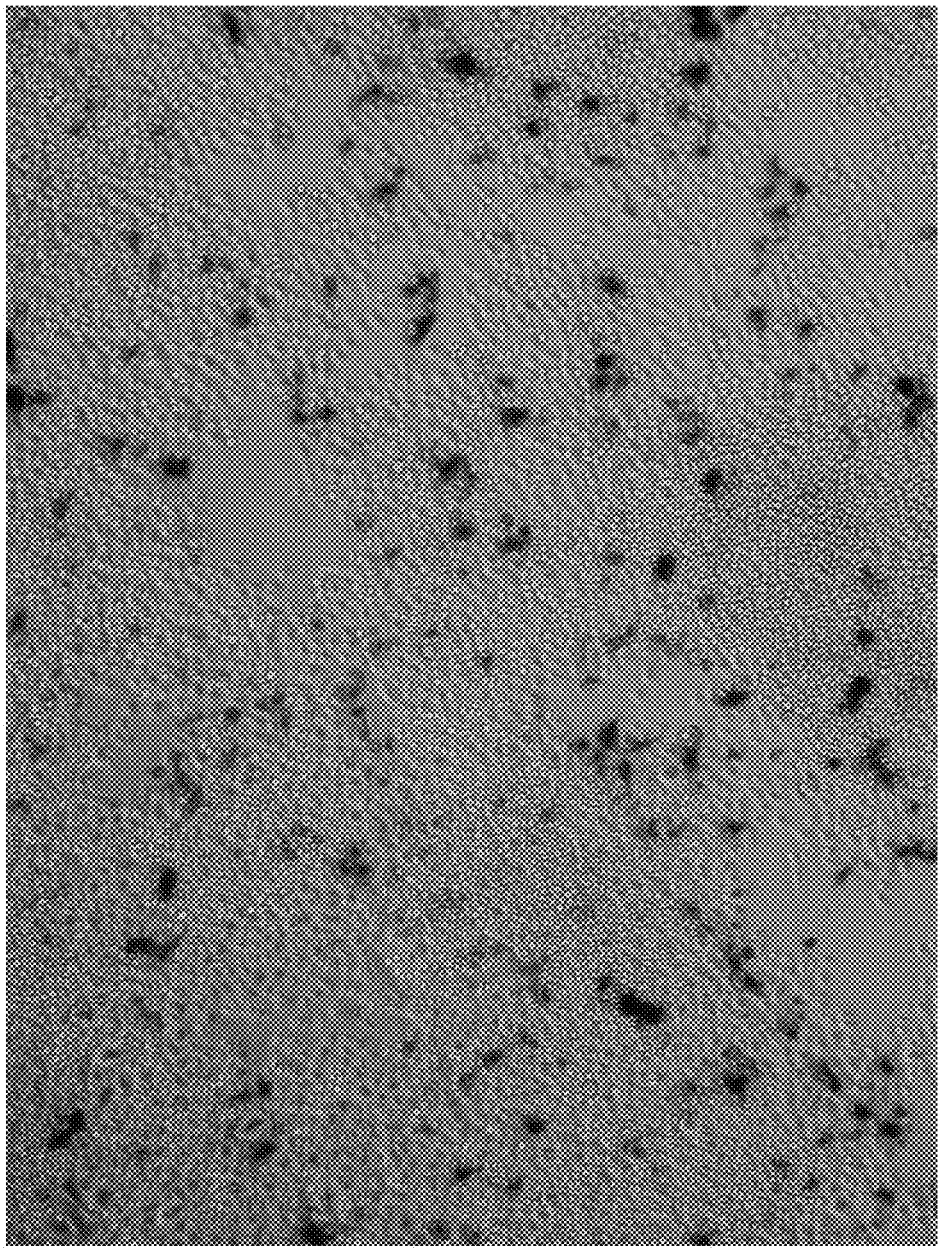


FIG. 9

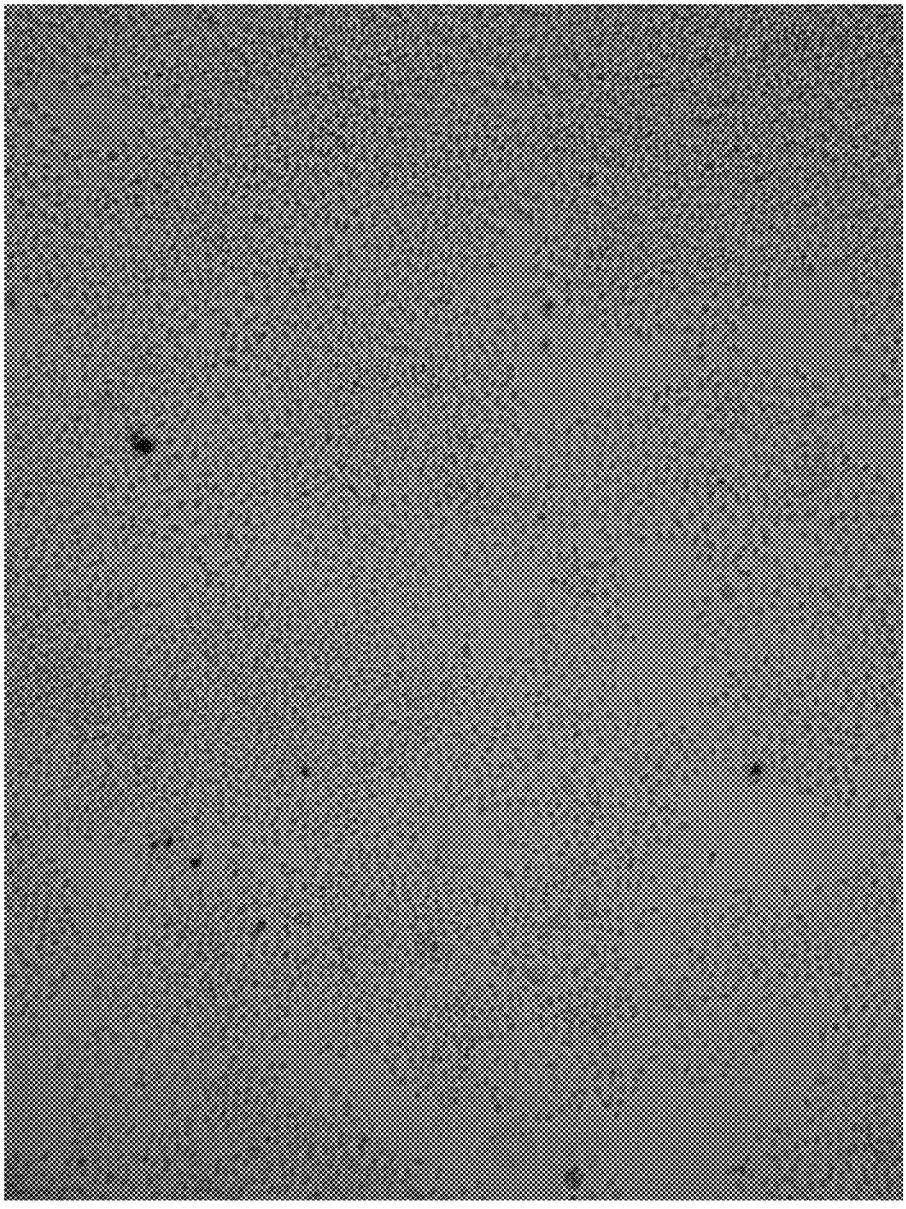


FIG. 10

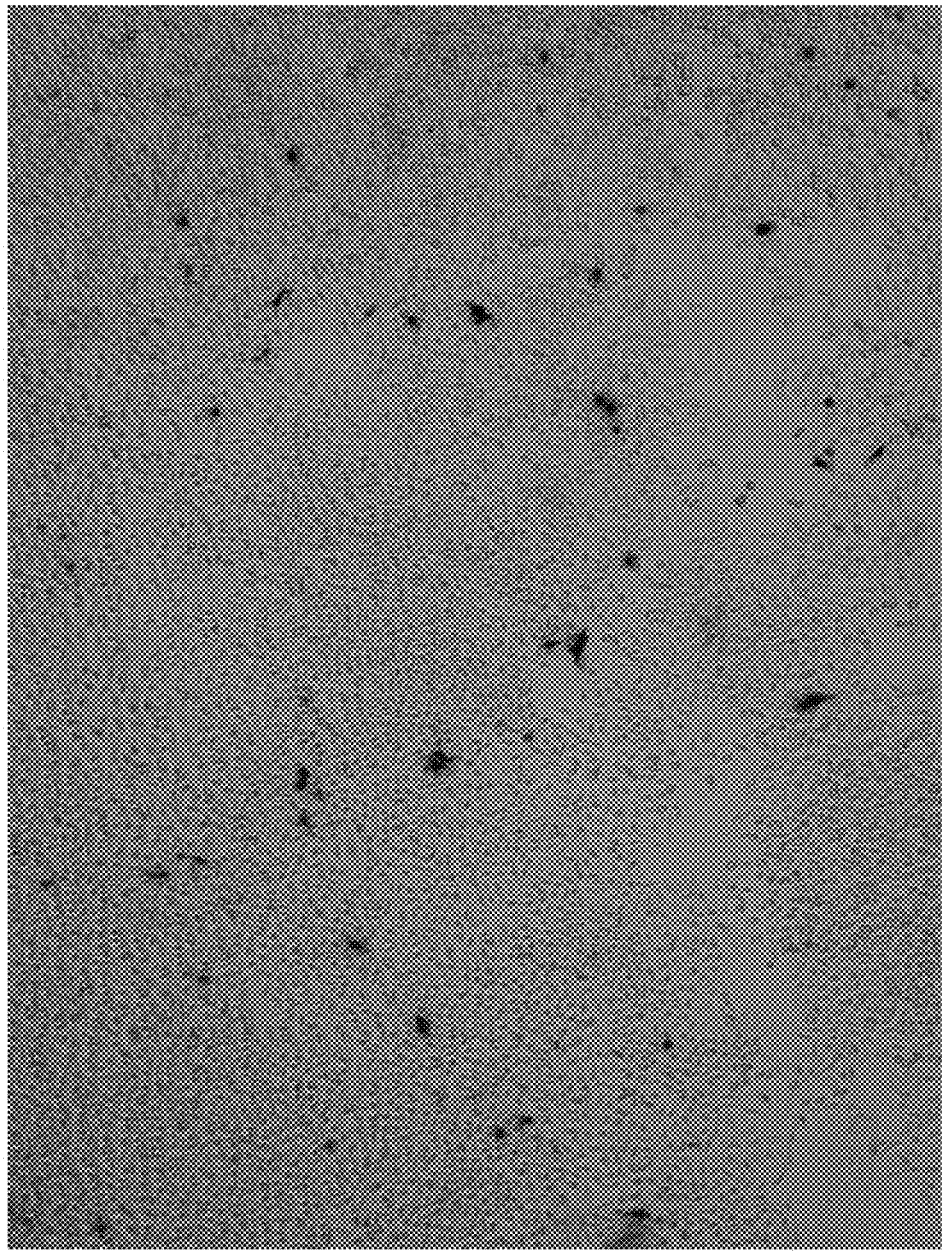


FIG. 11

FIG. 12

# METHODS AND COMPOSITIONS RELATING TO IMPROVED LENTIVIRAL VECTOR PRODUCTION SYSTEMS

The present application is a continuation of U.S. patent application Ser. No. 12/578,346, filed Oct. 13, 2009, which is a divisional of U.S. patent application Ser. No. 10/209,952, filed Aug. 1, 2002, now U.S. Pat. No. 7,629,153, which claims the benefit of U.S. Provisional Application No. 60/309,569, filed Aug. 2, 2001, the entire text of each of which is herein incorporated by reference.

## BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to improved lentiviral vectors, their production and their safe use in gene delivery and expression of desired transgenes in target cells.

2. Description of Related Art

Transfection of cells is an increasingly important method of delivering gene therapy and nucleic acid based treatment for a number of disorders. Transfection is the introduction of nucleic acids into recipient eukaryotic cells and the subsequent integration of the nucleic acid sequence into chromosomal DNA. Efficient transfection requires vectors, which facilitate the introduction of foreign nucleic acids into the desired cells, may provide mechanisms for chromosomal integration, and provide for the appropriate expression of the traits or proteins encoded by those nucleic acids. The design and construction of efficient, reliable, and safe vectors for cell transfection continues to be a substantial challenge to gene therapy and treatment methods.

Viruses of many types have formed the basis for vectors. Virus infection involves the introduction of the viral genome into the host cell. That property is co-opted for use as a gene delivery vehicle in viral based vectors. The viruses used are often derived from pathogenic viral species that already have many of the necessary traits and abilities to transfect cells. However, not all viruses will successfully transfect all cell types at all stages of the cell cycle. Thus, in the development of viral vectors, viral genomes are often modified to enhance their utility and effectiveness for introducing foreign gene constructs (transgenes) or other nucleic acids. At the same time, modifications may be introduced that reduce or eliminate their ability to cause disease.

Lentiviruses are a subgroup of retroviruses that can infect nondividing cells owing to the karyophilic properties of their preintegration complex, which allow for its active import through the nucleopore. Correspondingly, lentiviral vectors derived from human immunodeficiency virus type 1 (HIV-1) can mediate the efficient delivery, integration and long-term expression of transgenes into non-mitotic cells both in vitro and in vivo (Naldini et al., 1996a; Naldini et al., 1996b; Blomer et al., 1997). For example, HIV-based vectors can efficiently transduce human $CD34^+$ hematopoietic cells in the absence of cytokine stimulation (Akkina et al., 1996; Sutton et al., 1998; Uchida et al., 1998; Miyoshi et al., 1999; Case et al., 1999), and these cells are capable of long-term engraftment in NOD/SCID mice (Miyoshi et al., 1999). Furthermore, bone marrow from these primary recipients can repopulate secondary mice with transduced cells, confirming the lentivector-mediated genetic modification of very primitive hematopoietic precursors, most probably bona fide stem cells. Since none of the other currently available gene delivery systems has such an ability, lentiviral vectors provide a previously unexplored basis for the study of hematopoiesis and similar phenomena, and for the gene therapy of inherited and acquired disorders via the genetic modification of human stem cells (HCLs).



This important capability is subject to significant biosafety concerns (Akkina et al., 1996; Sutton et al., 1998; Uchida et al., 1998). The accidental generation of replication-competent recombinants (RCRs) during the production of lentiviral vector stocks represents one of the major problems to be solved before lentiviral vectors can be considered for human gene therapy.

In the retroviral genome, a single RNA molecule that also contains all the necessary cis-acting elements carries all the coding sequences. Biosafety of a vector production system is therefore best achieved by distributing the sequences encoding its various components over as many independent units as possible, to maximize the number of crossovers that would be required to re-create an RCR. Lentivector particles are generated by co-expressing the virion packaging elements and the vector genome in host producer cells, e.g. 293 human embryonic kidney cells. In the case of HIV-1-based vectors, the core and enzymatic components of the virion come from HIV-1, while the envelope protein is derived from a heterologous virus, most often VSV due to the high stability and broad tropism of its G protein. The genomic complexity of HIV, where a whole set of genes encodes virulence factors essential for pathogenesis but dispensable for transferring the virus genetic cargo, substantially aids the development of clinically acceptable vector systems.

Multiply attenuated packaging systems typically now comprise only three of the nine genes of HIV-1: gag, encoding the virion main structural proteins, pol, responsible for the retrovirus-specific enzymes, and rev, which encodes a post-transcriptional regulator necessary for efficient gag and pol expression (Dull, et al., 1998). From such an extensively deleted packaging system, the parental virus cannot be reconstituted, since some 60% of its genome has been completely eliminated. In one version of an HIV-based packaging system, Gag/Pol, Rev, VSV G and the vector are produced from four separate DNA units. Also, the overlap between vector and helper sequences has been reduced to a few tens of nucleotides so that opportunities for homologous recombination are minimized.

HIV type 1 (HIV-1) based vector particles may be generated by co-expressing the virion packaging elements and the vector genome in a so-called producer cell, e.g. 293T human enbryonic kidney cells. These cells may be transiently transfected with a number of plasmids. Typically from three to four plasmids are employed, but the number may be greater depending upon the degree to which the lentiviral components are broken up into separate units. Generally, one plasmid encodes the core and enzymatic components of the virion, derived from HIV-1. This plasmid is termed the packaging plasmid. Another plasmid encodes the envelope protein(s), most commonly the G protein of vesicular stomatitis virus (VSV G) because of its high stability and broad tropism. This plasmid may be termed the envelope expression plasmid. Yet another plasmid encodes the genome to be transferred to the target cell, that is, the vector itself, and is called the transfer vector. Recombinant viruses with titers of several millions of transducing units per milliliter (TU/ml) can be generated by this technique and variants thereof. After ultracentrifugation concentrated stocks of approximately $10^9$ TU/ml can be obtained.

The vector itself is the only genetic material transferred to the target cells. It typically comprises the transgene cassette flanked by cis-acting elements necessary for its encapsidation, reverse transcription, nuclear import and integration. As

has been previously done with oncoretroviral vectors, lentiviral vectors have been made that are "self-inactivating" in that they lose the transcriptional capacity of the viral long terminal repeat (LTR) once transferred to target cells (Zufferey, et al. 1998). This modification further reduces the risk of emergence of replication competent recombinants (RCR) and avoids problems linked to promoter interference.

Nevertheless, experience with retroviral vectors demonstrates that the emergence of a replication-competent retrovirus (RCR) is possible, although a rare event even when vectors are produced by stable packaging cell lines and components designed to provide high safety. The pathogenic potential of RCRs is demonstrated by the induction of cancer in monkeys injected with contaminated oncoretroviral vector stocks. Consequently, the administration of retroviral vectors to human patients is authorized only if the presence of contaminant RCRs has been excluded by a test sensitive enough to detect a single RCR in an aliquot equal to 5% of the dose actually used. Creating highly safe vectors is clearly important when doses equal or superior to $10^{10}$ transducing units may be necessary to reach therapeutic efficiency.

There is therefore a significant need to develop improved lentiviruses for use as transducing vectors capable of effectively transducing cells and expressing desired transgenes at high levels while meeting biosafety requirements. Currently available lentiviral vector production systems rely on the expression of packaging and vector elements either by transient transfection or in stable cell lines. Deletion of non-essential genes from the parental virus and splitting of the vector system components on separate DNA units act to help minimize the risk of emergence of RCRs. Greatest safety is achieved with the fewest, or, ideally, with zero RCR occurrence in vector production. The present invention utilizes specific changes in the packaging and vector system components, their methods of production and their methods of use in order to further reduce or eliminate the occurrence of RCR.

## SUMMARY OF THE INVENTION

The present invention provides for compositions and methods that improve the biosafety of lentiviral vector production systems in such a way that, if its components undergo multiple recombination events reconstituting the parental virus, the resulting recombinant will still be defective with respect to the ability to proceed through subsequent infection and replication. The invention further improves the biosafety of lentiviral vector production by optionally providing for drug sensitivity for any resulting recombinants.

The present invention thus concerns, in a general and overall sense, improved vectors and methods for the production thereof that are designed to permit the safe transfection and transduction of animal cells, particularly human cells, and more particularly hematopoietic progenitor cells, or stem cells (hHSC). The present invention facilitates appropriate expression of desired transgenes in such cells by providing effective vectors with increased safety.

The viral vectors of the present invention, therefore, may be generally described as recombinant vectors that include at least the lentiviral gag and pol genes, that is, those genes required for virus production, which permit their manufacture in reasonable quantities using available producer cell lines. To meet important human safety needs, the more preferred vectors in accordance with the present invention will not include any other active lentiviral genes, such as vpr, vif, vpu, nef, tat, such as where these genes have been removed or otherwise inactivated. In fact, it is preferred that the only active lentiviral genes present in the vector will be at most the aforementioned gag and pol genes, supplemented by the rev gene as may be required for efficient cytoplasmic export and expression of vector genes.

The most preferred lentiviral genes and cis-acting sequence elements (e.g., long terminal repeats or LTRs, the psi signal, the RRE) used in preparing lentivectors in accordance with the present invention will be one that is human immunodeficiency virus (HIV) derived, and more particularly, HIV-1 derived. Thus, the gag, pol and rev genes will preferably be HIV genes and more preferably HIV-1 genes. However, the gag, pol and rev genes and cis-acting sequence elements from other lentiviruses may be employed for certain applications in accordance with the present invention, including the genes and cis-acting sequence elements of HIV-2, simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), bovine immunodeficiency virus (BIV), Jembrana Disease Virus (JDV), equine infectious anemia virus (EIAV), caprine arthritis encephalitis virus (CAEV) and the like. Such constructs could be useful, for example, where one desires to modify certain cells of non-human origin. However, the HIV based vector backbones (i.e., HIV cis-acting sequence elements and HIV gag, pol and rev genes) will generally be preferred in connection with most aspects of the present invention in that HIV-based constructs are the most efficient at transduction of human cells.

The most preferred configuration of the packaging elements is one in which the gag, pol and rev genes are present. However, the need for rev may be alleviated in some designs by using cis-acting sequences facilitating the cytoplasmic export of incompletely spliced RNAs in the absence of Rev, the so-called constitutive RNA export element or CTE such as found in Mason-Pfizer monkey virus (Bray et al., 1994). Alternatively, specific codons may be altered in the gag and pol genes to similar effect (Kotsopoulou, et al., 2000). Also, components of the pol gene such as the integrase can be provided in a trans configuration, for instance as a VPR-integrase fusion protein (Wu, et al., 2000).

In the lentivectors of the present invention it is particularly desirable to employ mutations in the central polypurine tract (cPPT) of the sequence encoding the lentiviral Gag/Pol polyprotein in the packaging plasmid such that the introduced mutations interfere with lentiviral replication relative to wild-type genome. Such constructs provide a biosafety feature in that the nuclear import of replication-competent recombinants. This feature greatly minimizes the risk that (RCRs) will emerge. The cPPT/cTS region need be inactive only on the packaging plasmid construct to confer this safety feature. Indeed, an active copy of the cPPT/cTS region is typically provided on the transfer vector plasmid.

It is also desirable to employ in the present invention an additional sequence element in the packaging plasmid encoding the lentiviral Gag/Pol polyprotein in order to increase the genome length of any potential recombinant lentiviruses such that the effects of mutation in the central polypurine tract are maximized. This feature also minimizes the risk of producing RCRs. The long sequence element may be introduced into the vector genome at various positions that provide for maximizing the effects of the mutations in the central polypurine tract of the sequence encoding the lentiviral Gag/Pol polyprotein. A particularly preferred position is between the end of the pol or gag genes and the beginning of the RRE sequence element.

In another preferred aspect of the invention, such long sequence elements encode one or more genes conferring susceptibility to drugs currently used with success to treat viral infection. One such sequence element may include sequence that encodes a thymidinekinase, or the IRES-tk cassette. One

skilled in the art will recognize, of course, that any such drug susceptibility gene or genes, or any like construct may be employed to similar effect.

In a further preferred aspect of the invention, the 5' LTR R-U5 region of the vector plasmid contains a set of mutations that additionally prevent the replication of putative viral recombinants. Such mutations preferably include changes that either destabilize or excessively stabilize the Poly(A) hairpin motif, which leads to reduced replication of any RCRs.

One of skill in the art will recognize that the ultimate efficacy of these various aspects of the invention will depend upon the particular combination of aspects employed. It is preferred that the mutant sequences of the Poly(A) hairpin structures in the 5' LTR R-U5 region of the vector plasmid are to be used in conjunction with other preferred aspects. It is also contemplated that the invention may be embodied as various combinations of the individually described embodiments, including a combination of all disclosed embodiments, only two of the disclosed embodiments, or, employed singly in the making and using of such lentiviral vectors in the transfection and transduction of cells. In a most preferred embodiment of the present invention all these aspects of the present invention will be present.

The present invention describes gene transfer vehicles that appear particularly well suited for the transduction of cells and for the expression of transgenes in various cell types. These compositions and methods will facilitate the safe use of lentiviral vectors for the genetic manipulation of cells, and should be particularly useful for both research and therapeutic applications.

It will be understood by the skilled artisan that the invention is not limited to any one particular cell type and that one may use the lentiviral vectors and methods of the invention for the expression of transgenes in many cell types. Some examples of cell types contemplated include terminally differentiated cells such as neurons, lung cells, muscle cells, liver cells, pancreatic cells, endothelial cells, cardiac cells, skin cells, bone marrow stromal cells, ear and eye cells. Additionally, stem cells and progenitor cells such as pancreatic ductal cells, neural precursors, and mesodermal stem cells are also contemplated. Most notably, however, the more preferred lentivectors of the present invention have highly desirable features that permit the high level expression of transgenes in human progenitor cells while meeting human biosafety requirements.

It is believed that the lentivectors of the present invention may be employed to deliver any transgene that one desires, depending on the application. In the case of delivery to hematopoietic progenitor cells, one will typically select a transgene that will confer a desirable function on such cells, including, for example, globin genes, hematopoietic growth factors, which include erythropoietin (EPO), the interleukins (such as Interleukin-1 (IL-1), Interleukin-2 (IL-2), Interleukin-3 (IL-3), Interleukin-6 (IL-6), Interleukin-12 (IL-12), etc.) and the colony-stimulating factors (such as granulocyte colony-stimulating factor, granulocyte/macrophage colony-stimulating factor, or stem-cell colony-stimulating factor), the platelet-specific integrin αIIbβ, multidrug resistance genes, the gp91 or gp 47 genes that are defective in patients with chronic granulomatous disease (CGD), antiviral genes rendering cells resistant to infections with pathogens such as human immunodeficiency virus, genes coding for blood coagulation factors VIII or IX which are mutated in hemophiliacs, ligands involved in T cell-mediated immune responses such as T cell antigen receptors, B cell antigen receptors (immunoglobulins), the interleukin receptor common y chain, as well as combination of T and B cell antigen receptors alone or in combination with single chain antibodies such as ScFv, tumor necrosis factor (TNF), IL-2, IL-12, gamma interferon, CTLA4, B7 and the like, genes expressed in tumor cells such as Melana, MAGE genes (such as MAGE-1, MAGE-3), P198, P1A, gp100 etc.

A principal application of the present invention will be to provide for vectors that deliver desired transgenes to hematopoietic cells for a number of possible reasons. This might include, but of course not be limited to, the treatment of myelosupression and neutropenias which may be caused as a result of chemotherapy or immunosupressive therapy or infections such as AIDS, genetic disorders, cancers and the like.

Exemplary genetic disorders of hematopoietic cells that are contemplated include sickle cell anemia, thalassemias, hemaglobinopathies, Glanzmann thrombasthenia, lysosomal storage disorders (such as Fabry disease, Gaucher disease, Niemann-Pick disease, and Wiskott-Aldrich syndrome), severe combined immunodeficiency syndromes (SCID), as well as diseases resulting from the lack of systemic production of a secreted protein, for example, coagulation factor VIII and/or IX. In such cases, one would desire to introduce transgenes such as globin genes, hematopoietic growth factors, which include erythropoietin (EPO), the interleukins (especially Interleukin-1, Interleukin-2, Interleukin-3, Interleukin-6, Interleukin-12, etc.) and the colony-stimulating factors (such as granulocyte colony-stimulating factor, granulocyte/macrophage colony-stimulating factor, or stem-cell colony-stimulating factor), the platelet-specific integrin αIIbβ, multidrug resistance genes, the gp91 or gp 47 genes which are defective in patients with chronic granulomatous disease (CGD), antiviral genes rendering cells resistant to infections with pathogens such as human immunodeficiency virus, genes coding for blood coagulation factors VIII or IX which are mutated in hemophiliacs, ligands involved in T cell-mediated immune responses such as T cell antigen receptors, B cell antigen receptors (immunoglobulins), the interleukin receptor common y chain, as well as combination of T and B cell antigen receptors alone and/or in combination with single chain antibodies (ScFv), IL2, IL12, TNF, gamma interferon, CTLA4, B7 and the like, genes expressed in tumor cells such as Melana, MAGE genes (such as MAGE-1, MAGE-3), P198, P1A, gp100 etc.

Exemplary cancers are those of hematopoietic origin, for example, arising from myeloid, lymphoid or erythroid lineages, or precursor cells thereof. Exemplary myeloid disorders include, but are not limited to, acute promyeloid leukemia (APML), acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML). Lymphoid malignancies which may be treated utilizing the lentivectors of the present invention include, but are not limited to acute lymphoblastic leukemia (ALL) which includes B-lineage ALL and T-lineage ALL, chronic lymphocytic leukemia (CLL), prolymphocytic leukemia (PLL), hairy cell leukemia (HLL) and Waldenstrom's macroglobulinemia (WM). Additional forms of malignant lymphomas contemplated as candidates for treatment utilizing the lentiviral vectors of the present invention include, but are not limited to non-Hodgkin lymphoma and variants thereof, peripheral T-cell lymphomas, adult T-cell leukemia/lymphoma (ATL), cutaneous T-cell lymphoma (CTCL), large granular lymphocytic leukemia (LGF) and Hodgkin's disease.

In other embodiments, the present invention is directed to host cells that have been transduced with one of the foregoing lentivectors. It is believed that the lentivectors of the present invention can be employed to transduce most any cell. Exem-

plary cells include but are not limited to a $CD4^+$ T cell, a peripheral blood lymphocyte cell, a peripheral blood mononuclear cell, a hematopoietic stem cell, a fetal cord blood cell, a fibroblast cell, a brain cell, a lung cell, a liver cell, a muscle cell, a pancreatic cell, an endothelial cell, a cardiac cell, a skin cell, a bone marrow stromal cell, and an eye cells, a pancreatic ductal cell, a neural precursor, a mesodermal stem cell and the like. The cells transduced may further be primate, murine, porcine, or human in origin, or come from another animal species.

For the production of virus particles, one may employ any cell that is compatible with the expression of lentiviral Gag and Pol genes, or any cell that can be engineered to support such expression. For example, producer cells such as 293T cells, TE 671 and HT1080 cells may be used.

Of course, as noted above, the lentivectors of the invention will be particularly useful in the transduction of human hematopoietic progenitor cell or a hematopoietic stem cell, obtained either from the bone marrow, the peripheral blood or the umbilical cord blood, as well as in the tranduction of a $CD4^+$ T cell, a peripheral blood B or T lymphocyte cell, a peripheral blood mononuclear cell, a dendritic cell, and a monocytic cell. Particularly preferred targets are $CD34^+$ cells.

In still other embodiments, the present invention is directed to a method for transducing a human hematopoietic stem cell comprising contacting a population of human cells that include hematopoietic stem cells with one of the foregoing lentivectors under conditions to effect the transduction of a human hematopoietic progenitor cell in said population by the vector. The stem cells may be transduced in vivo or in vitro, depending on the ultimate application. Even in the context of human gene therapy, such as gene therapy of human stem cells, one may transduce the stem cell in vivo or, alternatively, transduce in vitro followed by infusion of the transduced stem cell into a human subject. In one aspect of this embodiment, the human stem cell can be removed from a human, e.g., a human patient, using methods well known to those of skill in the art and transduced as noted above. The transduced stem cells are then reintroduced into the same or a different human.

Where a human subject is treated directly by introduction of the vector into the subject, the treatment is typically carried out by intravenous administration of the vector. When cells, for instance $CD34^+$ cells, dendritic cells, peripheral blood cells or tumor cells are transduced ex vivo, the vector particles are incubated with the cells using a dose generally in the order of between 1 to 50 multiplicities of infection (MOI) which also corresponds to $1\times10^5$ to $50\times10^5$ transducing units of the viral vector per $10^5$ cells. This of course includes amount of vector corresponding to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, and 50 MOI. Typically, the amount of vector may be expressed in terms of HeLa transducing units (TU). Other routes for vector administration include intrarterially, endoscopically, intralesionally, percutaneously, subcutaneously, intramuscular, intrathecally, intraorbitally, intradermally, intraperitoneally, transtracheally, subcuticularly, by intrastemal injection, by inhalation or intranasal spraying, by endotracheal route and the like. In embodiments concerning tumor/cancer therapies with the vectors of the invention the expression vector can be delivered by direct injection into the tumor or into the tumor vasculature.

A typical example of ex vivo gene therapy is a patient suffering from chronic granulatous disease (CGD), whose $CD34^+$ cells can be isolated from the bone marrow or the peripheral blood and transduced ex vivo with a lentivector expressing the gp91phox gene before reimplantation. In the case of patients suffering from severe combined immunodeficiency (SCID), the inventors contemplate a similar approach, using lentivectors of the invention expressing the gene defective in the patient, for example, the gene encoding the common gamma chain of the Interleukin receptor. For the genetic treatment of HIV infection, the present inventors contemplate intracellular immunization, wherein cells are rendered resistant to the HIV virus through the introduction of antiviral genes. In embodiments of the intracellular immunization for HIV, targets of the lentivectors of the invention include hematopoietic progenitors, peripheral blood $CD4^+$ T cells, and monocytes. As will be recognized by the skilled artisan, similar intracellular immunization methods can be used for other viral infections as well. For the immunotherapy of cancers, tumor cells or antigen presenting cells such as dendritic cells will be genetically engineered with the lentivectors of the invention. For cancer therapies some transgenes that may be used in the lentivector constructs of the invention are those that can inhibit, and/or kill, and/or prevent the proliferation, and/or mediate the apoptosis of, the cancer/tumor cell and/or genes such as TNF.

The lentivectors described herein may also be used in vivo, by direct injection into the blood or into a specific organ. For example, in one embodiment intracerebral injection of lentivectors expressing the Glial Cell Derived Nerve Growth Factor (GDNF), can be used for the treatment of Parkinson's disease. In another example, intraportal injection of a lentivector expressing coagulation factor VIII for the correction of hemophilia A is envisioned. In yet another example, intravenous or intramuscular injection of a lentivector of the present invention expressing the dystrophin gene for the treatment of Duchenne Muscular Dystrophy is envisioned. Thus, one of ordinary skill in the art will appreciate the extensive use of the lentivector constructs of the present invention in terms of gene therapies.

As used herein the specification or claim(s) when used in conjunction with the word "comprising", the words "a" or "an" may mean one or more than one. As used herein "another" may mean at least a second or more.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

## BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. **1**. Schematic drawing of pMDLg/pRRE and pMDLD. The cPPT/cTS sequence element (black box) is indicated on the pol gene of pMDLg/pRRE. The plasmid pMDLD is a modified version with multiple mutations in the cPTT sequence element abolishing its function. Sequence comparison between the two plasmids is shown at the bottom.

FIG. **2**. Inactivation of the cPPT sequence element in the packaging system does not affect vector production. Vectors transducing GFP were produced in parallel with packaging systems having or lacking a functional cPPT sequence ele-

ment by transient transfection of 293T cells. Vector stocks were matched for their reverse transcriptase activity and used to transduce 293T cells. Two days later, the percentage of GFP positive cells was determined by FACS. Vector titers were identical whether the packaging system had a functional or a mutated cPPT.

FIG. **3**. Strategies to increase the length of packaging plasmids. To maximize the benefit of the cPPT inactivation, genome length of recombinant lentiviruses must be as long as possible. To this end DNA sequence can be inserted between the pol gene and the RRE sequence element. The inserted DNA can work either as a stuffer only or may be chosen to fulfill additional functions. One possibility is the use of a gene conferring drug sensitivity to cells infected by the recombinant lentiviruses e.g. the thymidinekinase gene (tk) from the Herpes simplex virus (HSV). An internal ribosomal entry site (IRES) is placed upstream of the tk gene to allow its efficient expression.

FIG. **4**. Mutations known for their strong inhibitory effect on HIV-1 replication were introduced in the R-U5 region of HIV-1 based vectors transducing the GFP gene.

FIG. **5**. Mutations in the R-U5 region of lentivirus vectors do not compromise their transduction efficiency. Vector production and determination of GFP positive cells were as in FIG. **2**. Mutation C was found not to affect the transduction efficacy of the vector whereas Mutation A decreases the apparent titer of the vector by a factor 10. However, the lower number of GFP positive cells with the Mut A vector reflects the fact that the mutation prevents polyadenylation at the viral LTR but does not indicate a low transduction efficacy. This point is demonstrated in FIG. **7**.

FIG. **6**. Since the presence of MutA inhibits the function of the viral polyadenylation signal, the MutA was tested in a vector carrying its own polyadenylation signal, pA, and the GFP gene. Mut A vectors carrying their own polyadenylation signal function as wild-type vectors.

FIG. **7**. Apparent titers of wild-type and vectors carrying MutA and a polyadenylation signal (pA) were identical. Vector production and determination of GFP positive cells were as in FIG. **2**.

FIG. **8**. Sequence and secondary structure of mutations A and C with the entire panel of six disclosed by Das et al. (1997).

FIG. **9**. Infectivity of wild-type HIV-1 in HeLa cells. Colored cells indicate successful infection. Each colored cell corresponds to one infection event.

FIG. **10**. Substantially reduced infectivity conferred by an inactive cPPT/cTS region in conjunction with a wild-type genome length. Viral titers were adjusted so as to equalize reverse transcriptase activity to those used in FIG. **9**.

FIG. **11**. Infectivity conferred by an inactive cPPT/cTS region in conjunction with a genome length 1470 shorter than wild type. Viral titers were adjusted so as to equalize reverse transcriptase activity to those used in FIGS. **9** and **10**.

FIG. **12**. Background staining of cells in the absence of virus. The absence of colored cells indicates the lack of false positives in the assays that produced FIGS. **9**, **10**, and **11**.

## SEQUENCE SUMMARY

SEQ ID NO:1 corresponds to positions 5296 to 5760 of the plasmid pMDL g/p RRE, derived from the HIV-1 molecular clone NL4-3 (Accession number M19921) but modified to inactivate the cPPT/cTS region. The resulting sequence differs from the wild-type in the cPPT/cTS region, positions 5432 through 5452 as indicated in FIG. **1** and described in SEQ ID NO:4. SEQ ID NO:2 and SEQ ID NO:3 correspond to nucleotide positions 5954 through 6558, inclusive, of previously a described vector, pHR'-CMVLacZ, (Accession number AF105229), but incorporate the nucleotide sequence changes as described by Das, et al. (1997). The sequences contain Eco RV and Bss HII restriction enzyme sites at the 5' and 3' ends, respectively, which are useful in introducing the sequences into desired constructs. SEQ ID NO:5 and SEQ ID NO:6 are the sequences of the poly(A) hairpin structures that substantially inhibit viral replication as identified in FIG. **8** and described in Das, et al. (1997), and which are contained within SEQ ID NO:2 and SEQ ID NO:3, respectively.

## DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

While lentiviral vectors offer a great potential for gene-therapy and especially the transduction of human hematopoietic stem cells (hHSC), vectors developed so far still suffer from concerns regarding their biosafety. The present invention overcomes such and other deficiencies in the art and describes the development of HIV-derived vectors that have improved biosafety characteristics.

The present invention provides HIV-derived vectors which are safe, highly efficient, and very potent for expressing transgenes in human and animal cells, including but not limited to hematopoietic progenitor cells as well as in all other blood cell derivatives. These vectors therefore provide useful tools for genetic treatments such as inherited and acquired disorders, gene-therapies for cancers especially the hematological cancers, as well as for the study of hematopoiesis via lentivector-mediated modification of human HSCs.

### A. LENTIVIRAL VECTORS AND GENE THERAPY

Lentiviruses are complex retroviruses, which, in addition to the common retroviral genes gag, pol, and env, contain other genes with regulatory or structural function. The higher complexity enables the virus to modulate its life cycle, as in the course of latent infection. Some examples of lentivirus include the Human Immunodeficiency Viruses: HIV-1, HIV-2 and the Simian Immunodeficiency Virus: SIV. Lentiviral vectors have been generated by multiply attenuating the HIV virulence genes, for example, the genes env, vif, vpr, vpu, nef and tat are deleted making the vector biologically more safe.

Lentiviral vectors offer great advantages for gene therapy. They integrate stably into chromosomes of target cells which is required for long-term expression. Further, they do not transfer viral genes therefore avoiding the problem of generating transduced cells that can be destroyed by cytotoxic T-cells. Furthermore, they have a relatively large cloning capacity, sufficient for most envisioned clinical applications. In addition, lentiviruses, in contrast to other retroviruses, are capable of transducing non-dividing cells. This is very important in the context of gene-therapy for tissues such as the hematopoietic system, the brain, liver, lungs and muscle. For example, vectors derived from HIV-1 allow efficient in vivo and ex vivo delivery, integration and stable expression of transgenes into cells such a neurons, hepatocytes, and myocytes (Blomer et al., 1997; Kafri et al., 1997; Naldini et al., 1996; Naldini et al., 1998).

The lentiviral genome and the proviral DNA have the three genes found in retroviruses: gag, pol and env, which are flanked by two long terminal repeat (LTR) sequences. The gag gene encodes the internal structural (matrix, capsid and nucleocapsid) proteins; the pol gene encodes the RNA-directed DNA polymerase (reverse transcriptase), a protease

and an integrase; and the env gene encodes viral envelope glycoproteins. The 5' and 3' LTR's serve to promote transcription and polyadenylation of the virion RNAs, respectively. Lentiviruses have additional genes including vif, vpr, tat, rev, vpu, nef and vpx.

Adjacent to the 5' LTR are sequences necessary for reverse transcription of the genome (the tRNA primer binding site) and for efficient encapsidation of viral RNA into particles (the Psi site). If the sequences necessary for encapsidation (or packaging of retroviral RNA into infectious virions) are missing from the viral genome, the cis defect prevents encapsidation of genomic RNA. However, the resulting mutant remains capable of directing the synthesis of all virion proteins.

Lentiviral vectors are known in the art, see Naldini et al., (1996a, 1996b, and 1998); Zufferey et al., (1997); Dull et al., 1998, U.S. Pat. Nos. 6,013,516; and 5,994,136 all incorporated herein by reference. In general, these vectors are plasmid-based or virus-based, and are configured to carry the essential sequences for incorporating foreign nucleic acid, for selection and for transfer of the nucleic acid into a host cell.

Two components are involved in making a virus-based gene delivery system: first, the packaging elements, encompassing the structural proteins as well as the enzymes necessary to generate an infectious particle, and second, the vector itself, i.e., the genetic material to be transferred. Biosafety safeguards can be introduced in the design of both of these components. Thus, the packaging unit of the first generation HIV-based vectors comprised all HIV-1 proteins except the envelope protein (Naldini et al., 1998, 1996a). Subsequently it was shown that the deletion of four additional viral genes that are responsible for virulence including, vpr, vif, vpu and nef did not alter the utility of the vector system (Zufferey et al., 1997). It was also shown that Tat, the main transactivator of HIV is also dispensable for the generation of a fully efficient vector (Dull et al., 1998). Thus, the third-generation packaging unit of the HIV-based lentiviral vectors comprise only three genes of the parental virus: gag, pol and rev, which helps to eliminate the possibility of reconstitution of a wild-type virus through recombination.

This system was further improved by removing HIV transcriptional units from the vector (Zufferey et al., 1998). It was demonstrated therein that introducing a deletion in the U3 region of the 3' LTR of the DNA used to produce the vector RNA generated self-inactivating (SIN) vectors. During reverse transcription this deletion is transferred to the 5' LTR of the proviral DNA. Enough sequence was eliminated, including the removal of a TATA box, which abolished the transcriptional activity of the LTR, which prevents production of full-length vector RNA in transduced cells. This however did not affect vector titers or the in vitro or in vivo properties of the vector.

The present invention provides several improvements to the existing lentivectors as described above and in other parts of this specification. Introducing a lentivector providing a heterologous gene, such as genes to treat hematopoietic and lympho-hematopoietic disorders in this invention, into a packaging cell yields a producer cell which releases infectious vector particles carrying the foreign gene of interest.

The env gene can be derived from any virus, including retroviruses. The env preferably is an amphotropic envelope protein which allows transduction of cells of human and other species. Examples of retroviral-derived env genes include, but are not limited to: Moloney murine leukemia virus (MoMuLV or MMLV), Harvey murine sarcoma virus (HaMuSV or HSV), murine mammary tumor virus (MuMTV or MMTV), gibbon ape leukemia virus (GaLV or GALV), human immunodeficiency virus (HIV) and Rous sarcoma virus (RSV). Other env genes such as Vesicular stomatitis virus (VSV) protein G (VSV G), that of hepatitis viruses and of influenza also can be used.

While VSV G protein is a desirable env gene because VSV G confers broad host range on the recombinant virus, VSV G can be deleterious to the host cell, e.g. the packaging cell. Thus, when a gene such as that for VSV G is used, it is preferred to employ an inducible promoter system so that VSV G expression can be regulated to minimize host toxicity when VSV G is expression is not required. For example, the tetracycline-regulated gene expression system of Gossen & Bujard, (1992) can be employed to provide for inducible expression of VSV G when tetracycline is withdrawn from the transferred cell. Thus, the tetNP16 transactivator is present on a first vector and the VSV G coding sequence is cloned downstream from a promoter controlled by tet operator sequences on another vector.

The vector providing the viral env nucleic acid sequence is associated operably with regulatory sequences, e.g., a promoter or enhancer. The regulatory sequence can be any eukaryotic promoter or enhancer, including for example, EF1$\alpha$, PGK, the Moloney murine leukemia virus promoter-enhancer element, the human cytomegalovirus enhancer, the vaccinia P7.5 promoter or the like (also see examples listed in Tables 1 and 2 below). In some cases, such as the Moloney murine leukemia virus promoter-enhancer element, the promoter-enhancer elements are located within or adjacent to the LTR sequences. Preferably, the regulatory sequence is one which is not endogenous to the lentivirus from which the vector is being constructed. Thus, if the vector is being made from SIV, the SIV regulatory sequence found in the SIV LTR would be replaced by a regulatory element which does not originate from SIV.

One may further target the recombinant virus by linkage of the envelope protein with an antibody or a particular ligand for targeting to a receptor of a particular cell-type. By inserting a sequence (including a regulatory region) of interest into the viral vector, along with another gene which encodes the ligand for a receptor on a specific target cell, for example, the vector is now target-specific. Retroviral vectors can be made target-specific by inserting, for example, a glycolipid or a protein. Targeting often is accomplished by using an antigen-binding portion of an antibody or a recombinant antibody-type molecule, such as a single chain antibody, to target the retroviral vector. Those of skill in the art will know of, or can readily ascertain without undue experimentation, specific methods to achieve delivery of a retroviral vector to a specific target.

The heterologous or foreign nucleic acid sequence, such as a polynucleotide sequence encoding a gene such as a therapeutic gene for inherited or acquired hematopoietic disorders herein, is linked operably to a regulatory nucleic acid sequence. Preferably, the heterologous sequence is linked to a promoter, resulting in a chimeric gene.

Marker genes may be utilized to assay for the presence of the vector, and thus, to confirm infection and integration. The presence of a marker gene ensures the selection and growth of only those host cells which express the inserts. Typical selection genes encode proteins that confer resistance to antibiotics and other toxic substances, e.g., histidinol, puromycin, hygromycin, neomycin, methotrexate, and cell surface markers.

The recombinant virus of the invention is capable of transferring a nucleic acid sequence into a mammalian cell. The term, "nucleic acid sequence", refers to any nucleic acid molecule, preferably DNA, as discussed in detail herein. The nucleic acid molecule may be derived from a variety of

sources, including DNA, cDNA, synthetic DNA, RNA or combinations thereof. Such nucleic acid sequences may comprise genomic DNA which may or may not include naturally occurring introns. Moreover, such genomic DNA may be obtained in association with promoter regions, poly A sequences or other associated sequences. Genomic DNA may be extracted and purified from suitable cells by means well known in the art. Alternatively, messenger RNA (mRNA) can be isolated from cells and used to produce cDNA by reverse transcription or other means.

The vectors are introduced via transfection or infection into the packaging cell line. The packaging cell line produces viral particles that contain the vector genome. Methods for transfection or infection are well known by those of skill in the art. After cotransfection of the packaging vectors and the transfer vector to the packaging cell line, the recombinant virus is recovered from the culture media and tittered by standard methods used by those of skill in the art. Thus, the packaging constructs can be introduced into human cell lines by calcium phosphate transfection, lipofection or electroporation, generally together with a dominant selectable marker, such as neomycin, DHFR, Glutamine synthetase or ADA, followed by selection in the presence of the appropriate drug and isolation of clones. The selectable marker gene can be linked physically to the packaging genes in the construct.

Stable cell lines wherein the packaging functions are configured to be expressed by a suitable packaging cell are known. For example, see U.S. Pat. No. 5,686,279; and Ory et al., (1996), which describe packaging cells. The packaging cells with a lentiviral vector incorporated in them form producer cells. Producer cells are thus cells or cell-lines that can produce or release packaged infectious viral particles carrying the therapeutic gene of interest. These cells can further be anchorage dependent which means that these cells will grow, survive, or maintain function optimally when attached to a surface such as glass or plastic. The producer cells may also be neoplastically transformed cells. Some examples of anchorage dependent cell lines used as lentiviral vector packaging cell lines when the vector is replication competent are HeLa or 293 cells and PERC.6 cells.

In some applications, particularly when the virus is to be used for gene therapy applications, it is preferable that the vector be replication deficient (or replication defective) to avoid uncontrolled proliferation of the virus in the individual to be treated. In such instances mammalian cell lines are selected which have been engineered, either by modification of the producer cell's genome to encode essential viral functions or by the co-infection of the producer cell with a helper virus, to express proteins complementing the effect of the sequences deleted from the viral genome. For example, for HIV-1 derived vectors, the HIV-1 packaging cell line, PSI422, may be used as described in Corbeau, et al. (1996). Similarly, where the viral vector to be produced is a retrovirus, the human 293-derived retroviral packaging cell line (293GPG) capable of producing high titers of retroviral particles may be employed as described in Ory, et al. (1996). In the production of minimal vector systems, the producer cell is engineered (either by modification of the viral genome or by the use of helper virus or cosmid) to complement the functions of the parent virus enabling replication and packaging into virions in the producer cell line.

Lentiviral transfer vectors Naldini et al., (1996), have been used to infect human cells growth-arrested in vitro and to transduce neurons after direct injection into the brain of adult rats. The vector was efficient at transferring marker genes in vivo into the neurons and long term expression in the absence of detectable pathology was achieved. Animals analyzed ten months after a single injection of the vector showed no decrease in the average level of transgene expression and no sign of tissue pathology or immune reaction (Blomer et al., 1997).

## B. THE CPPT/CTS REGION

The introduction of foreign nucleic acids into the nucleus of a cell requires importation of the nucleic acids into the nucleus through the nuclear membrane. Lentiviruses utilize an active nuclear import system, which forms the basis of their ability to replicate efficiently in non-dividing cells. This active import system relies upon a complex series of events including a specific modality for reverse transcription. In particular, in HIV-1, the central polypurine tract (cPPT), located within the pol gene, initiates synthesis of a downstream plus strand while plus strand synthesis is also initiated at the 3' polypurine tract (PPT). After strand transfer of the short DNA molecule, the upstream plus strand synthesis will initiate and proceed until the center of the genome is reached. At the central termination sequence (cTS) the HIV-1 reverse transcriptase is ejected, (released from its template), when functioning in a strand displacement mode. (Charneau, et al., 1994) The net result is a double stranded DNA molecule with a stable flap, 99 nucleotides in length at the center of the genome.

This central "flap" facilitates nuclear import. (Zennou, et al., 2000). Defects in the cPPT/cTS region that prevent the efficient reverse transcription initiating at the cPPT/cTS region prevent the formation of the central DNA flap. The resulting DNA molecules accumulate as non-integrated linear viral DNA outside the nucleus. (Zennou, et al., 2000). Thus, an inactive, or substantially less active cPPT/cTS region in a lentiviral vector packaging construct, if reconstituted into an RCR, will prevent efficient nuclear import of the RCR DNA genome during any subsequent steps towards infection. The absence of a DNA flap in an HIV-1 virus system severely impairs viral DNA nuclear import. (Zennou, et al., 2000). Importantly, Zennou, et al. show that the addition of cPPT on the transfer vector increases levels of integration by a factor of five, whereas the inactivation of the cPPT on the viral genome itself decreases replication by several order of magnitude. Although unknown to Zennou, et al., this difference is a function of the shorter size of the vector compared to the viral genome.

The cPPT/cTS region acts in cis with the rest of the viral genome. The region extends over 118 nucleotides in HIV-1 and exists in similar form in other lentiviruses. The region is found at or near the center of all lentiviral genomes (Zennou, et al., 2000). The cPPT/cTS sequence element overlaps with the gene encoding the integrase protein and is present in an active form in all packaging systems described to date.

Wild-type activity of the cPPT/cTS region may be effectively eliminated by the mutation of the underlying nucleic acid sequence so as to disrupt the activity without effecting the function of the integrase protein, which is also encoded by that sequence and its surrounding sequence. Packaging plasmids so altered do not reduce the vector titers that may be achieved and so retain all the benefits of any vector production system in which they are incorporated.

The elimination of wild-type activity of the cPPT/cTS region from viral packaging systems improves their biosafety by preventing the efficient nuclear import of any RCR DNA genome during any subsequent steps towards infection. This protective effect of an inactive cPPT/cTS region may operate in any RCR lentiviral genome. However, the protective effects can be optimized or enhanced by incorporating into

the packaging plasmid a stuffer sequence, whose purpose is to enlarge the eventual genome size of any RCR that may be produced. Larger viral genomes are more dependent upon a fully functional cPPT/cTS region for entry into the nucleus. Thus, a larger genome size, at least the size of a wild-type lentivirus such as HIV-1, is less able to enter the nucleus through the mechanism mediated by the cPPT/cTS sequence region. Correspondingly, in lentiviral vectors packaging plasmids whose size has been shortened through the removal or modification of non-essential or virulence encoding genes, a stuffer sequence may be inserted to enlarge the genome size, thus utilizing more effectively the protective effects of an inactive or mutant cPPT/cTS region.

The stuffer sequence need not be of any particular sequence other than one which does not rescue infectivity or in any other way contribute to virulence of any possible RCRs that might be generated. The sequence should be of a size, however, to increase the protective effects of inactive or mutant cPPT/cTS regions. For a minimal packaging plasmid such as pMDLD a stuffer sequence of about 4.4 kb in size effectively recreates the native genome length of a lentivirus, and thus effectively augments the effects of mutant cPPT/cTS regions. Optimally, the stuffer sequence will be located between the pol gene and the RRE, a location that optimizes the likely effects of a larger genome size on the inhibition of nuclear import by mutant cPPT/cTS regions.

## C. DRUG SUSCEPTIBILITY

The biosafety benefits provided by the replication inhibitory effects of larger RCR genomes in conjunction with inactive cPPT/cTS regions may be further enhanced by employing drug susceptibility genes. Drug susceptibility genes encode proteins whose presence results in any virus incorporating/expressing the genes being susceptible to therapeutic drugs. Thus, any unintended RCR infection may be specifically and effectively treated.

The stuffer sequence may encode such drug susceptibility genes. One particular sequence that confers drug susceptibility is the thymidine kinase gene (Zhao-Emonet et al., 1999). The expression of a drug susceptibility gene such as thymidine kinase may be driven by a promoter. One such promoter is the IRES element. Further details of the IRES element and its use as a promoter is provided below. In the current context, the IRES promoter and a thymidine kinase gene may be provided as an expression cassette, which may be inserted into the packaging plasmid as the "IRES-tk" cassette. The insertion of the IRES-tk cassette provides both for a genome length that aids in the effectiveness of the modifications to the cPPT/cTS region and provides a "suicide" gene that allows therapeutic treatment of any infection with RCRs (one treat the infected patient, not the RCR itself) that are produced and infective (Zhao-Emonet et al., 1999). The tk gene of the IRES-tk cassette is derived from the Herpes simplex virus and confers susceptibility to the drug Ganciclovir, a substrate of TK. Thus, if an RCR is generated that is capable of infection, the resulting infected cells may be killed with Ganciclovir, thereby preventing the further spread of the RCR. Similarly, cells expressing the cytosine deaminase gene can be killed with 5-fluorocytosine (Greco, 2001).

## D. THE POLY(A) HAIRPIN

The 5' untranslated leader sequences of lentiviral genomes contain several sequence elements crucial for viral replication. These include elements essential for transcription, mRNA splicing, dimerization, packaging, and reverse transcription. Much of the function of the regions depends upon the secondary structure of the viral RNA (Das, et al., 1997). One such structure is a hairpin that comprises the polyadenylation signal (AAUAAA). The structure is therefore known as the poly(A) hairpin. The poly(A) hairpin is part of the R-U5 domain of the LTR and is present in both the 5' and 3' ends of the proviral genome of lentiviruses.

The role of the poly(A) hairpin in replication activity has been conserved despite the divergence in sequence among the various lentiviruses (Das, et al., 1997). Disruption of the poly(A) hairpin structure through mutation of the sequence involved severely inhibits replication activity (Das, et al., 1997). Mutant sequences of the 5' LTR poly(A) hairpin region can induce such defects in replication if they are such that they either sufficiently destabilize the hairpin or act to excessively stabilize the hairpin. For an efficient hairpin structure, the thermodynamic stability of the sequence pairings must remain within a relatively narrow limits (Das, et al., 1997).

Das, et al., (1997), incorporated herein by reference, created several different mutations within the 5' LTR poly(A) region of HIV-1 and evaluated the effects of those sequence mutations on replication activity. Mutant A of Das, et al. (1997) stabilized the hairpin structure to an extent sufficient to substantially inhibit wild-type replication activity. Mutant C of Das, et al. (1997) destabilized the hairpin structure and also substantially inhibited replication activity. The sequences of Mutant A and Mutant C are provided herein as SEQ ID NO:2 and SEQ ID NO:5 for Mutant A, and SEQ ID NO:3 and SEQ ID NO:6 for Mutant C, respectively.

Particular embodiments of the present invention may include providing a transfer vector incorporating the replication inhibiting 5' LTR poly(A) sequences of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:6. Preferably, these sequence elements are present in combination with one or more aspects of the embodiments described elsewhere in this specification. Thus, mutant 5' LTR poly(A) sequences may be incorporated into a transfer vector that is to be used in conjunction with a packaging plasmid incorporating the cPPT/cTS region displaying reduced replication activity. Further, the transfer vector of the present invention may be used in conjunction with a packaging plasmid containing a stuffer sequence to further maximize the effects of the mutated cPPT/cTS regions so employed. As indicated elsewhere in this specification, such a stuffer sequence may encode drug susceptibility genes or expression cassettes for drug susceptibility. All these aspects of the present invention will be present in a most preferred embodiment of the present invention.

## E. SIN DESIGN

The SIN design further increases the biosafety of lentiviral vectors. A majority of the HIV LTR is comprised of the U3 sequences. The U3 region contains the enhancer and promoter elements that modulate basal and induced expression of the HIV genome in infected cells and in response to cell activation. Several of these promoter elements are essential for viral replication. Some of the enhancer elements are highly conserved among viral isolates and have been implicated as critical virulence factors in viral pathogenesis. The enhancer elements may act to influence replication rates in the different cellular target of the virus (Marthas et al., 1993).

As viral transcription starts at the 3' end of the U3 region of the 5' LTR, those sequences are not part of the viral mRNA and a copy thereof from the 3' LTR acts as template for the generation of both LTR's in the integrated provirus. If the 3' copy of the U3 region is altered in a retroviral vector con-

struct, the vector RNA is still produced from the intact 5' LTR in producer cells, but cannot be regenerated in target cells. Transduction of such a vector results in the inactivation of both LTR's in the progeny virus. Thus, the retrovirus is self-inactivating (SIN) and those vectors are known as SIN transfer vectors.

The SIN design is described in further detail in Zufferey et al., 1998 and U.S. Pat. No. 5,994,136 both incorporated herein by reference. As described therein, there are, however, limits to the extent of the deletion at the 3' LTR. First, the 5' end of the U3 region serves another essential function in vector transfer, being required for integration. Thus, the terminal dinucleotide and att sequence may represent the 5' boundary of the U3 sequences which can be deleted. In addition, some loosely defined regions may influence the activity of the downstream polyadenylation site in the R region. Excessive deletion of U3 sequence from the 3' LTR may decrease polyadenylation of vector transcripts with adverse consequences both on the titer of the vector in producer cells and the transgene expression in target cells. On the other hand, limited deletions may not abrogate the transcriptional activity of the LTR in transduced cells.

The lentiviral vectors described herein carry deletions of the U3 region of the 3' LTR spanning from nucleotide −418 to −18. This is the most extensive deletion and extends as far as to the TATA box, therefore abrogating any transcriptional activity of the LTR in transduced cells. The titer of vector in producer cells as well as transgene expression in target cells was unaffected in these vectors. This design therefore provides an enormous increase in vector safety.

SIN-type vectors with such extensive deletions of the U3 region cannot be generated for murine leukemia virus (MLV) or spleen necrosis virus (SNV) based retroviral vectors without compromising efficiency of transduction.

Elimination of the −418 to −18 nucleotide sequence abolishes transcriptional activity of the LTR, thereby abolishing the production of full length vector RNA in transduced cells. In the HIV-derived lentivectors none of the in vitro or in vivo properties were compromised by the SIN design. Importantly, the additional biosafety features of the present invention may be incorporated into SIN-type vectors and non-SIN-type vectors with equal results.

## G. POSTTRANSCRIPTIONALLY REGULATING ELEMENTS (PRE)

Enhancing transgene expression may be required in certain embodiments, especially those that involve lentiviral constructs of the present invention with modestly active promoters.

One type of PRE is an intron positioned within the expression cassette, which can stimulate gene expression. However, introns can be spliced out during the life cycle events of a lentivirus. Hence, if introns are used as PRE's they may have to be placed in an opposite orientation to the vector genomic transcript.

Posttranscriptional regulatory elements that do not rely on splicing events offer the advantage of not being removed during the viral life cycle. Some examples are the posttranscriptional processing element of herpes simplex virus, the posttranscriptional regulatory element of the hepatitis B virus (HPRE) and the woodchuck hepatitis virus (WPRE). Of these the WPRE is most preferred as it contains an additional cis-acting element not found in the HPRE (Donello et al., 1998). This regulatory element is positioned within the vector so as to be included in the RNA transcript of the transgene, but downstream of stop codon of the transgene translational unit. As demonstrated in the present invention and in Zufferey et al., 1999, the WPRE element is a useful tool for stimulating and enhancing gene expression of desired transgenes in the context of the lentiviral vectors.

The WPRE is characterized and described in U.S. Pat. No. 6,136,597, incorporated herein by reference. As described therein, the WPRE is an RNA export element that mediates efficient transport of RNA from the nucleus to the cytoplasm. It enhances the expression of transgenes by insertion of a cis-acting nucleic acid sequence, such that the element and the transgene are contained within a single transcript. Presence of the WPRE in the sense orientation was shown to increase transgene expression by up to 7 to 10 fold. Retroviral vectors deliver sequences in the form of cDNAs instead of complete intron-containing genes as introns are generally spliced out during the sequence of events leading to the formation of the retroviral particle. Introns mediate the interaction of primary transcripts with the splicing machinery. Because the processing of RNAs by the splicing machinery facilitates their cytoplasmic export, due to a coupling between the splicing and transport machineries, cDNAs are often inefficiently expressed. Thus, the inclusion of the WPRE in a vector results in enhanced expression of transgenes.

## H. NUCLEIC ACIDS

One embodiment of the present invention is to transfer nucleic acids encoding a therapeutic gene, especially a gene that provides therapy for hematopoietic and lympho-hematopoietic disorders, such as the inherited or acquired disorders described above. In one embodiment the nucleic acids encode a full-length, substantially full-length, or functional equivalent form of such a gene.

Thus, in some embodiments of the present invention, the treatment of a hematopoietic and lympho-hematopoietic disorder involves the administration of a lentiviral vector of the invention comprising a therapeutic nucleic acid expression construct to a cell of hematopoietic origin. It is contemplated that the hematopoietic cells take up the construct and express the therapeutic polypeptide encoded by nucleic acid, thereby restoring the cells normal phenotype.

A nucleic acid may be made by any technique known to one of ordinary skill in the art. Non-limiting examples of synthetic nucleic acid, particularly a synthetic oligonucleotide, include a nucleic acid made by in vitro chemical synthesis using phosphotriester, phosphite or phosphoramidite chemistry and solid phase techniques such as described in EP 266,032, incorporated herein by reference, or via deoxynucleoside H-phosphonate intermediates as described by Froehler et al., 1986, and U.S. Pat. No. 5,705,629, each incorporated herein by reference. A non-limiting example of enzymatically produced nucleic acid include one produced by enzymes in amplification reactions such as PCR™ (see for example, U.S. Pat. No. 4,683,202 and U.S. Pat. No. 4,682,195, each incorporated herein by reference), or the synthesis of oligonucleotides described in U.S. Pat. No. 5,645,897, incorporated herein by reference. A non-limiting example of a biologically produced nucleic acid includes recombinant nucleic acid production in living cells (see for example, Sambrook et al. 1989, incorporated herein by reference).

A nucleic acid may be purified on polyacrylamide gels, cesium chloride centrifugation gradients, or by any other means known to one of ordinary skill in the art (see for example, Sambrook et al. 1989, incorporated herein by reference).

The term "nucleic acid" will generally refer to at least one molecule or strand of DNA, RNA or a derivative or mimic thereof, comprising at least one nucleobase, such as, for example, a naturally occurring purine or pyrimidine base found in DNA (e.g., adenine "A," guanine "G," thymine "T," and cytosine "C") or RNA (e.g. A, G, uracil "U," and C). The term "nucleic acid" encompasses the terms "oligonucleotide" and "polynucleotide." The term "oligonucleotide" refers to at least one molecule of between about 3 and about 100 nucleobases in length. The term "polynucleotide" refers to at least one molecule of greater than about 100 nucleobases in length. These definitions generally refer to at least one single-stranded molecule, but in specific embodiments will also encompass at least one additional strand that is partially, substantially or fully complementary to the at least one single-stranded molecule. Thus, a nucleic acid may encompass at least one double-stranded molecule or at least one triple-stranded molecule that comprises one or more complementary strand(s) or "complement(s)" of a particular sequence comprising a strand of the molecule.

In certain embodiments, a "gene" refers to a nucleic acid that is transcribed. As used herein, a "gene segment" is a nucleic acid segment of a gene. In certain aspects, the gene includes regulatory sequences involved in transcription, or message production or composition. In particular embodiments, the gene comprises transcribed sequences that encode for a protein, polypeptide or peptide. In other particular aspects, the gene comprises a nucleic acid, and/or encodes a polypeptide or peptide-coding sequences of a gene that is defective or mutated in a hematopoietic and lympho-hematopoietic disorder. In keeping with the terminology described herein, an "isolated gene" may comprise transcribed nucleic acid(s), regulatory sequences, coding sequences, or the like, isolated substantially away from other such sequences, such as other naturally occurring genes, regulatory sequences, polypeptide or peptide encoding sequences, etc. In this respect, the term "gene" is used for simplicity to refer to a nucleic acid comprising a nucleotide sequence that is transcribed, and the complement thereof. In particular aspects, the transcribed nucleotide sequence comprises at least one functional protein, polypeptide and/or peptide encoding unit. As will be understood by those in the art, this functional term "gene" includes both genomic sequences, RNA or cDNA sequences, or smaller engineered nucleic acid segments, including nucleic acid segments of a non-transcribed part of a gene, including but not limited to the non-transcribed promoter or enhancer regions of a gene. Smaller engineered gene nucleic acid segments may express, or may be adapted to express using nucleic acid manipulation technology, proteins, polypeptides, domains, peptides, fusion proteins, mutants and/or such like. Thus, a "truncated gene" refers to a nucleic acid sequence that is missing a stretch of contiguous nucleic acid residues.

Various nucleic acid segments may be designed based on a particular nucleic acid sequence, and may be of any length. By assigning numeric values to a sequence, for example, the first residue is 1, the second residue is 2, etc., an algorithm defining all nucleic acid segments can be created:

$$n \text{ to } n+y$$

where n is an integer from 1 to the last number of the sequence and y is the length of the nucleic acid segment minus one, where n+y does not exceed the last number of the sequence. Thus, for a 10-mer, the nucleic acid segments correspond to bases 1 to 10, 2 to 11, 3 to 12 . . . and/or so on. For a 15-mer, the nucleic acid segments correspond to bases 1 to 15, 2 to 16, 3 to 17 . . . and/or so on. For a 20-mer, the nucleic segments correspond to bases 1 to 20, 2 to 21, 3 to 22 . . . and/or so on.

The nucleic acid(s) of the present invention, regardless of the length of the sequence itself, may be combined with other nucleic acid sequences, including but not limited to, promoters, enhancers, polyadenylation signals, restriction enzyme sites, multiple cloning sites, coding segments, and the like, to create one or more nucleic acid construct(s). The overall length may vary considerably between nucleic acid constructs. Thus, a nucleic acid segment of almost any length may be employed, with the total length preferably being limited by the ease of preparation or use in the intended recombinant nucleic acid protocol.

The term "vector" is used to refer to a carrier nucleic acid molecule into which a nucleic acid sequence can be inserted for introduction into a cell where it can be replicated. Vectors of the present invention are lentivirus based as described above and in other parts of the specification. The nucleic acid molecules carried by the vectors of the invention encode therapeutic genes and will be used for carrying out gene-therapies. One of skill in the art would be well equipped to construct such a therapeutic vector through standard recombinant techniques (see, for example, Maniatis et al., 1988 and Ausubel et al., 1994, both incorporated herein by reference).

The term "expression vector" refers to any type of genetic construct comprising a nucleic acid coding for a RNA capable of being transcribed. In some cases, RNA molecules are then translated into a protein, polypeptide, or peptide. In other cases, these sequences are not translated, for example, in the production of antisense molecules or ribozymes. Expression vectors can contain a variety of "control sequences," which refer to nucleic acid sequences necessary for the transcription and possibly translation of an operably linked coding sequence in a particular host cell. In addition to control sequences that govern transcription and translation, vectors and expression vectors may contain nucleic acid sequences that serve other functions as well and are described below.

(a) Promoters and Enhancers

A "promoter" is a control sequence that is a region of a nucleic acid sequence at which initiation and rate of transcription are controlled. It may contain genetic elements at which regulatory proteins and molecules may bind, such as RNA polymerase and other transcription factors, to initiate the specific transcription a nucleic acid sequence. The phrases "operatively positioned," "operatively linked," "under control," and "under transcriptional control" mean that a promoter is in a correct functional location and/or orientation in relation to a nucleic acid sequence to control transcriptional initiation and/or expression of that sequence.

A promoter generally comprises a sequence that functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as, for example, the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation. Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have been shown to contain functional elements downstream of the start site as well. To bring a coding sequence "under the control of" a promoter, one positions the 5' end of the transcription initiation site of the transcriptional reading frame "downstream" of (i.e., 3' of) the chosen promoter. The "upstream" promoter stimulates transcription of the DNA and promotes expression of the encoded RNA.

The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either cooperatively or independently to activate transcription. A promoter may or may not be used in conjunction with an "enhancer," which refers to a cis-acting regulatory sequence involved in the transcriptional activation of a nucleic acid sequence.

A promoter may be one naturally associated with a nucleic acid sequence, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment and/or exon. Such a promoter can be referred to as "endogenous." Similarly, an enhancer may be one naturally associated with a nucleic acid sequence, located either downstream or upstream of that sequence. Alternatively, certain advantages will be gained by positioning the coding nucleic acid segment under the control of a recombinant or heterologous promoter, which refers to a promoter that is not normally associated with a nucleic acid sequence in its natural environment. A recombinant or heterologous enhancer refers also to an enhancer not normally associated with a nucleic acid sequence in its natural environment. Such promoters or enhancers may include promoters or enhancers of other genes, and promoters or enhancers isolated from any other virus, or prokaryotic or eukaryotic cell, and promoters or enhancers not "naturally occurring," i.e., containing different elements of different transcriptional regulatory regions, and/or mutations that alter expression. For example, promoters that are most commonly used in recombinant DNA construction include the β-lactamase (penicillinase), lactose and tryptophan (trp) promoter systems. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including PCR™, in connection with the compositions disclosed herein (see U.S. Pat. Nos. 4,683,202 and 5,928,906, each incorporated herein by reference). Furthermore, it is contemplated the control sequences that direct transcription and/or expression of sequences within non-nuclear organelles such as mitochondria, chloroplasts, and the like, can be employed as well. Control sequences comprising promoters, enhancers and other locus or transcription controlling/modulating elements are also referred to as "transcriptional cassettes".

Naturally, it will be important to employ a promoter and/or enhancer that effectively directs the expression of the DNA segment in the organelle, cell type, tissue, organ, or organism chosen for expression. Those of skill in the art of molecular biology generally know the use of promoters, enhancers, and cell type combinations for protein expression, (see, for example Sambrook et al., 1989, incorporated herein by reference). The promoters employed may be constitutive, tissue-specific, inducible, and/or useful under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous for gene therapy or for applications such as the large-scale production of recombinant proteins and/or peptides. The promoter may be heterologous or endogenous.

Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

Tables 1 lists non-limiting examples of elements/promoters that may be employed, in the context of the present invention, to regulate the expression of a RNA. Table 2 provides non-limiting examples of inducible elements, which are regions of a nucleic acid sequence that can be activated in response to a specific stimulus.

TABLE 1

Promoter and/or Enhancer

| Promoter/Enhancer | References |
|---|---|
| Immunoglobulin Heavy Chain | Banerji et al., 1983; Gilles et al., 1983; Grosschedl et al., 1985; Atchinson et al., 1986, 1987; Imler et al., 1987; Weinberger et al., 1984; Kiledjian et al., 1988; Porton et al; 1990 |
| Immunoglobulin Light Chain | Queen et al., 1983; Picard et al., 1984 |
| T-Cell Receptor | Luria et al., 1987; Winoto et al., 1989; Redondo et al; 1990 |
| HLA DQ a and/or DQ β | Sullivan et al., 1987 |
| β-Interferon | Goodbourn et al., 1986; Fujita et al., 1987; Goodbourn et al., 1988 |
| Interleukin-2 | Greene et al., 1989 |
| Interleukin-2 Receptor | Greene et al., 1989; Lin et al., 1990 |
| MHC Class II 5 | Koch et al., 1989 |
| MHC Class II HLA-DRa | Sherman et al., 1989 |
| β-Actin | Kawamoto et al., 1988; Ng et al.; 1989 |
| Muscle Creatine Kinase (MCK) | Jaynes et al., 1988; Horlick et al., 1989; Johnson et al., 1989 |
| Prealbumin (Transthyretin) | Costa et al., 1988 |
| Elastase I | Omitz et al., 1987 |
| Metallothionein (MTII) | Karin et al., 1987; Culotta et al., 1989 |
| Collagenase | Pinkert et al., 1987; Angel et al., 1987 |
| Albumin | Pinkert et al., 1987; Tronche et al., 1989, 1990 |
| α-Fetoprotein | Godbout et al., 1988; Campere et al., 1989 |
| γ-Globin | Bodine et al., 1987; Perez-Stable et al., 1990 |
| β-Globin | Trudel et al., 1987 |
| c-fos | Cohen et al., 1987 |
| c-HA-ras | Triesman, 1986; Deschamps et al., 1985 |
| Insulin | Edlund et al., 1985 |
| Neural Cell Adhesion Molecule (NCAM) | Hirsh et al., 1990 |
| $\alpha_1$-Antitrypsin | Latimer et al., 1990 |

TABLE 1-continued

| Promoter and/or Enhancer | |
|---|---|
| Promoter/Enhancer | References |
| H2B (TH2B) Histone | Hwang et al., 1990 |
| Mouse and/or Type I Collagen | Ripe et al., 1989 |
| Glucose-Regulated Proteins (GRP94 and GRP78) | Chang et al., 1989 |
| Rat Growth Hormone | Larsen et al., 1986 |
| Human Serum Amyloid A (SAA) | Edbrooke et al., 1989 |
| Troponin I (TN I) | Yutzey et al., 1989 |
| Platelet-Derived Growth Factor (PDGF) | Pech et al., 1989 |
| Duchenne Muscular Dystrophy | Klamut et al., 1990 |
| SV40 | Banerji et al., 1981; Moreau et al., 1981; Sleigh et al., 1985; Firak et al., 1986; Herr et al., 1986; Imbra et al., 1986; Kadesch et al., 1986; Wang et al., 1986; Ondek et al., 1987; Kuhl et al., 1987; Schaffner et al., 1988 |
| Polyoma | Swartzendruber et al., 1975; Vasseur et al., 1980; Katinka et al., 1980, 1981; Tyndell et al., 1981; Dandolo et al., 1983; de Villiers et al., 1984; Hen et al., 1986; Satake et al., 1988; Campbell and/or Villarreal, 1988 |
| Retroviruses | Kriegler et al., 1982, 1983; Levinson et al., 1982; Kriegler et al., 1983, 1984a, b, 1988; Bosze et al., 1986; Miksicek et al., 1986; Celander et al., 1987; Thiesen et al., 1988; Celander et al., 1988; Chol et al., 1988; Reisman et al., 1989 |
| Papilloma Virus | Campo et al., 1983; Lusky et al., 1983; Spandidos and/or Wilkie, 1983; Spalholz et al., 1985; Lusky et al., 1986; Cripe et al., 1987; Gloss et al., 1987; Hirochika et al., 1987; Stephens et al., 1987 |
| Hepatitis B Virus | Bulla et al., 1986; Jameel et al., 1986; Shaul et al., 1987; Spandau et al., 1988; Vannice et al., 1988 |
| Human Immunodeficiency Virus | Muesing et al., 1987; Hauber et al., 1988; Jakobovits et al., 1988; Feng et al., 1988; Takebe et al., 1988; Rosen et al., 1988; Berkhout et al., 1989; Laspia et al., 1989; Sharp et al., 1989; Braddock et al., 1989 |
| Gibbon Ape Leukemia Virus | Holbrook et al., 1987; Quinn et al., 1989 |

TABLE 2

| Inducible Elements | | |
|---|---|---|
| Element | Inducer | References |
| MT II | Phorbol Ester (TFA) Heavy metals | Palmiter et al., 1982; Haslinger et al., 1985; Searle et al., 1985; Stuart et al., 1985; Imagawa et al., 1987, Karin et al., 1987; Angel et al., 1987b; McNeall et al., 1989 |
| MMTV (mouse mammary tumor virus) | Glucocorticoids | Huang et al., 1981; Lee et al., 1981; Majors et al., 1983; Chandler et al., 1983; Lee et al., 1984; Ponta et al., 1984; Sakai et al.,1988 |
| β-Interferon | Poly(rI)x Poly(rc) | Tavernier et al., 1983 |
| Adenovirus 5 E2 | ElA | Imperiale et al., 1984 |
| Collagenase | Phorbol Ester (TPA) | Angel et al., 1987a |
| Stromelysin | Phorbol Ester (TPA) | Angel et al., 1987b |
| SV40 | Phorbol Ester (TPA) | Angel et al., 1987b |
| Murine MX Gene | Interferon, Newcastle Disease Virus | Hug et al., 1988 |
| GRP78 Gene | A23187 | Resendez et al., 1988 |
| α-2-Macroglobulin | IL-6 | Kunz et al., 1989 |
| Vimentin | Serum | Rittling et al., 1989 |
| MHC Class I Gene H-2κb | Interferon | Blanar et al., 1989 |
| HSP70 | ElA, SV40 Large T Antigen | Taylor et al., 1989, 1990a, 1990b |
| Proliferin | Phorbol Ester-TPA | Mordacq et al., 1989 |
| Tumor Necrosis Factor | PMA | Hensel et al., 1989 |

TABLE 2-continued

| Inducible Elements | | |
|---|---|---|
| Element | Inducer | References |
| Thyroid Stimulating Hormone α Gene | Thyroid Hormone | Chatterjee et al., 1989 |

The identity of tissue-specific promoters or elements, as well as assays to characterize their activity, is well known to those of skill in the art. Non-limiting examples of such regions include the human LIMK2 gene (Nomoto et al., 1999), the somatostatin receptor 2 gene (Kraus et al., 1998), murine epididymal retinoic acid-binding gene (Lareyre et al., 1999), human CD4 (Zhao-Emonet et al., 1998), mouse alpha2 (XI) collagen (Tsumaki, et al., 1998), DIA dopamine receptor gene (Lee, et al., 1997), insulin-like growth factor II (Wu et al., 1997), and human platelet endothelial cell adhesion molecule-1 (Almendro et al., 1996).

The lentiviral vectors of the present invention are designed, primarily, to transfect cells with a therapeutic gene under the control of regulated eukaryotic promoters. Although the EF1α-promoter and the PGK promoter are preferred other promoter and regulatory signal elements as described in the Tables 1 and 2 above may also be used. Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of structural genes encoding the therapeutic gene of interest that is used in context with the lentiviral vectors of the present invention. Alternatively, a tissue-specific promoter for cancer gene therapy or the targeting of tumors may be employed with the lentiviral vectors of the present invention for treatment of cancers, especially hematological cancers.

Typically promoters and enhancers that control the transcription of protein encoding genes in eukaryotic cells are composed of multiple genetic elements. The cellular machinery is able to gather and integrate the regulatory information conveyed by each element, allowing different genes to evolve distinct, often complex patterns of transcriptional regulation.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Aside from this operational distinction, enhancers and promoters are very similar entities.

Promoters and enhancers have the same general function of activating transcription in the cell. They are often overlapping and contiguous, often seeming to have a very similar modular organization. Taken together, these considerations suggest that enhancers and promoters are homologous entities and that the transcriptional activator proteins bound to these sequences may interact with the cellular transcriptional machinery in fundamentally the same way.

A signal that may prove useful is a polyadenylation signal (hGH, BGH, SV40). The use of internal ribosome binding sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5'-methylated cap-dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well as an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message. In particular, the IRES element may be used to drive the expression of drug susceptibility genes such as thymidine kinase and the like.

In any event, it will be understood that promoters are DNA elements which when positioned functionally upstream of a gene leads to the expression of that gene. Most transgenes that will be introduced using the lentiviral vectors of the present invention are functionally positioned downstream of a promoter element.

A specific initiation signal also may be required for efficient translation of coding sequences. These signals include the ATG initiation codon or adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that the initiation codon must be "in-frame" with the reading frame of the desired coding sequence to ensure translation of the entire insert. The exogenous translational control signals and initiation codons can be either natural or synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements.

(b) Multiple Cloning Sites

Vectors of the present invention can include a multiple cloning site (MCS), which is a nucleic acid region that contains multiple restriction enzyme sites, any of which can be used in conjunction with standard recombinant technology to digest the vector (see, for example, Carbonelli et al., 1999, Levenson et al., 1998, and Cocea, 1997, incorporated herein by reference.) "Restriction enzyme digestion" refers to catalytic cleavage of a nucleic acid molecule with an enzyme that functions only at specific locations in a nucleic acid molecule. Many of these restriction enzymes are commercially available. Use of such enzymes is widely understood by those of skill in the art. Frequently, a vector is linearized or fragmented using a restriction enzyme that cuts within the MCS to enable exogenous sequences to be ligated to the vector. "Ligation" refers to the process of forming phosphodiester bonds between two nucleic acid fragments, which may or may not be contiguous with each other. Techniques involving restriction enzymes and ligation reactions are well known to those of skill in the art of recombinant technology.

(c) Splicing Sites

Most transcribed eukaryotic RNA molecules will undergo RNA splicing to remove introns from the primary transcripts. Vectors containing genomic eukaryotic sequences may require donor and/or acceptor splicing sites to ensure proper processing of the transcript for protein expression (see, for example, Chandler et al., 1997, herein incorporated by reference.)

(d) Termination Signals

The vectors or constructs of the present invention will generally comprise at least one termination signal. A "termination signal" or "terminator" is comprised of the DNA sequences involved in specific termination of an RNA transcript by an RNA polymerase. Thus, in certain embodiments a termination signal that ends the production of an RNA transcript is contemplated. A terminator may be necessary in vivo to achieve desirable message levels.

In eukaryotic systems, the terminator region may also comprise specific DNA sequences that permit site-specific cleavage of the new transcript so as to expose a polyadenylation site. This signals a specialized endogenous polymerase to add a stretch of about 200 A residues (polyA) to the 3' end of the transcript. RNA molecules modified with this polyA tail appear to be more stable and are translated more efficiently. Thus, in other embodiments involving eukaryotes, it is preferred that that terminator comprises a signal for the cleavage of the RNA, and it is more preferred that the terminator signal promotes polyadenylation of the message. The terminator and/or polyadenylation site elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

Terminators contemplated for use in the invention include any known terminator of transcription described herein or known to one of ordinary skill in the art, including but not limited to, for example, the termination sequences of genes, such as for example the bovine growth hormone terminator or viral termination sequences, such as for example the SV40 terminator. In certain embodiments, the termination signal may be a lack of transcribable or translatable sequence, such as due to a sequence truncation.

(e) Polyadenylation Signals

In eukaryotic gene expression, one will typically include a polyadenylation signal to effect proper polyadenylation of the

transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed. Some examples include the SV40 polyadenylation signal or the bovine growth hormone polyadenylation signal, convenient and known to function well in various target cells. Polyadenylation may increase the stability of the transcript or may facilitate cytoplasmic transport.

(f) Origins of Replication

In order to propagate a vector of the invention in a host cell, it may contain one or more origins of replication sites (often termed "ori"), which is a specific nucleic acid sequence at which replication is initiated. Alternatively an autonomously replicating sequence (ARS) can be employed if the host cell is yeast.

(g) Selectable and Screenable Markers

In certain embodiments of the invention, cells transduced with the lentivectors of the present invention may be identified in vitro or in vivo by including a marker in the expression vector. Such markers would confer an identifiable change to the transduced cell permitting easy identification of cells containing the expression vector. Generally, a selectable marker is one that confers a property that allows for selection. A positive selectable marker is one in which the presence of the marker allows for its selection, while a negative selectable marker is one in which its presence prevents its selection. An example of a positive selectable marker is a drug resistance marker.

Usually the inclusion of a drug selection marker aids in the cloning and identification of transfected cells, for example, genetic constructs that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful selectable markers. In addition to markers conferring a phenotype that allows for the discrimination of transformants based on the implementation of conditions, other types of markers including screenable markers such as GFP, whose basis is colorimetric analysis, are also contemplated. Alternatively, screenable enzymes such as herpes simplex virus thymidine kinase (tk) or chloramphenicol acetyltransferase (CAT) may be utilized. One of skill in the art would also know how to employ immunologic markers, possibly in conjunction with FACS analysis. The marker used is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding a gene product. Further examples of selectable and screenable markers are well known to one of skill in the art.

## I. HOST CELLS

As used herein, the terms "cell," "cell line," and "cell culture" may be used interchangeably. All of these terms also include their progeny, which is any and all subsequent generations. It is understood that all progeny may not be identical due to deliberate or inadvertent mutations. In the context of expressing a heterologous nucleic acid sequence, "host cell" refers to a prokaryotic or eukaryotic cell, and it includes any transformable organisms that is capable of replicating a vector and/or expressing a heterologous nucleic acid encoded by the vectors of this invention. A host cell can, and has been, used as a recipient for vectors. A host cell may be "transfected" or "transformed," which refers to a process by which exogenous nucleic acid is transferred or introduced into the host cell. A transformed cell includes the primary subject cell and its progeny. As used herein, the terms "engineered" and "recombinant" cells or host cells are intended to refer to a cell into which an exogenous nucleic acid sequence, such as, for example, a lentivector of the invention bearing a therapeutic gene construct, has been introduced. Therefore, recombinant cells are distinguishable from naturally occurring cells which do not contain a recombinantly introduced nucleic acid.

In certain embodiments, it is contemplated that RNAs or proteinaceous sequences may be co-expressed with other selected RNAs or proteinaceous sequences in the same host cell. Co-expression may be achieved by co-transfecting the host cell with two or more distinct recombinant vectors. Alternatively, a single recombinant vector may be constructed to include multiple distinct coding regions for RNAs, which could then be expressed in host cells transfected with the single vector.

Host cells may be derived from prokaryotes or eukaryotes, depending upon whether the desired result is replication of the vector or expression of part or all of the vector-encoded nucleic acid sequences. Numerous cell lines and cultures are available for use as a host cell, and they can be obtained through the American Type Culture Collection (ATCC), which is an organization that serves as an archive for living cultures and genetic materials (www.atcc.org). Some examples of host cells used in this invention include but are not limited to virus packaging cells, virus producer cells, 293T cells, human hematopoietic progenitor cells, human hematopoietic stem cells, $CD34^+$ cells, $CD4^+$ cells, and the like.

(a) Tissues and Cells

A tissue may comprise a host cell or cells to be transformed or contacted with a nucleic acid delivery composition and/or an additional agent. The tissue may be part or separated from an organism. In certain embodiments, a tissue and its constituent cells may comprise, but is not limited to, blood (e.g., hematopoietic cells, such as human hematopoietic progenitor cells, human hematopoietic stem cells, $CD34^+$ cells, $CD4^+$ cells, lymphocytes and other blood lineage cells), bone marrow, brain, stem cells, blood vessel, liver, lung, bone, breast, cartilage, cervix, colon, cornea, embryonic, endometrium, endothelial, epithelial, esophagus, facia, fibroblast, follicular, ganglion cells, glial cells, goblet cells, kidney, lymph node, muscle, neuron, ovaries, pancreas, peripheral blood, prostate, skin, small intestine, spleen, stomach, testes.

(b) Organisms

In certain embodiments, the host cell or tissue may be comprised in at least one organism. In certain embodiments, the organism may be, human, primate or murine. In other embodiments the organism may be any eukaryote or even a prokayote (e.g., a eubacteria, an archaea), as would be understood by one of ordinary skill in the art (see, for example, webpage http://phylogeny.arizona.edu/tree/phylogeny.html). Some lentivectors of the invention may employ control sequences that allow them to be replicated and/or expressed in both prokaryotic and eukaryotic cells. One of skill in the art would further understand the conditions under which to incubate all of the above described host cells to maintain them and to permit replication of a vector. Also understood and known are techniques and conditions that would allow large-scale production of the lentivectors of the invention, as well as production of the nucleic acids encoded by the lentivectors and their cognate polypeptides, proteins, or peptides some of which are therapeutic genes or proteins which will be used for gene therapies.

## J. INJECTABLE COMPOSITIONS AND PHARMACEUTICAL FORMULATIONS

To achieve gene-therapy using the lentiviral vector compositions of the present invention, one would generally contact a cell in need thereof with a lentiviral vector comprising

a therapeutic gene. The cell will further be in an organism such as a human in need of the gene therapy. The routes of administration will vary, naturally, with the location and nature of the disease, and include, e.g., intravenous, intrarterial, intradermal, transdermal, intramuscular, intranasal, subcutaneous, percutaneous, intratracheal, intraperitoneal, intratumoral, perfusion and lavage. The cells will also sometimes be isolated from the organisms, exposed to the lentivector ex vivo, and reimplanted afterwards.

Injection of lentiviral nucleic acid constructs of the invention may be delivered by syringe or any other method used for injection of a solution, as long as the expression construct can pass through the particular gauge of needle required for injection. A novel needleless injection system has recently been described (U.S. Pat. No. 5,846,233) having a nozzle defining an ampule chamber for holding the solution and an energy device for pushing the solution out of the nozzle to the site of delivery. A syringe system has also been described for use in gene therapy that permits multiple injections of predetermined quantities of a solution precisely at any depth (U.S. Pat. No. 5,846,225).

Solutions of the nucleic acids as free base or pharmacologically acceptable salts may be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions (U.S. Pat. No. 5,466,468, specifically incorporated herein by reference in its entirety). In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (e.g., glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intraarterial, intramuscular, subcutaneous, intratumoral and intraperitoneal administration. In this connection, sterile aqueous media that can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The compositions disclosed herein may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts, include the acid addition salts and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like.

As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The phrase "pharmaceutically-acceptable" or "pharmacologically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human. The preparation of an aqueous composition that contains a protein as an active ingredient is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared.

The terms "contacted" and "exposed," when applied to a cell, are used herein to describe the process by which a therapeutic lentiviral vector is delivered to a target cell.

For gene therapy to discrete, solid, accessible tumors, intratumoral injection, or injection into the tumor vasculature is specifically contemplated. Local, regional or systemic administration also may be appropriate. For tumors of >4 cm, the volume to be administered will be about 4-10 ml (preferably 10 ml), while for tumors of <4 cm, a volume of about 1-3 ml will be used (preferably 3 ml). Multiple injections delivered as single dose comprise about 0.1 to about 0.5 ml volumes. The viral particles may advantageously be contacted by administering multiple injections to the tumor, spaced at

approximately 1 cm intervals. Systemic administration is preferred for conditions such as hematological malignancies.

Continuous administration also may be applied where appropriate. Delivery via syringe or catherization is preferred. Such continuous perfusion may take place for a period from about 1-2 hours, to about 2-6 hours, to about 6-12 hours, to about 12-24 hours, to about 1-2 days, to about 1-2 wk or longer following the initiation of treatment. Generally, the dose of the therapeutic composition via continuous perfusion will be equivalent to that given by a single or multiple injections, adjusted over a period of time during which the perfusion occurs.

Treatment regimens may vary as well, and often depend on type of disease and location of diseased tissue, and factors such as the health and the age of the patient. The clinician will be best suited to make such decisions based on the known efficacy and toxicity (if any) of the therapeutic formulations based on lentiviral vectors of the present invention.

The treatments may include various "unit doses." A unit dose is defined as containing a predetermined-quantity of the therapeutic composition comprising a lentiviral vector of the present invention. The quantity to be administered, and the particular route and formulation, are within the skill of those in the clinical arts. A unit dose need not be administered as a single injection but may comprise continuous infusion over a set period of time. Unit dose of the present invention may conveniently be described in terms of transducing units (T.U.) of lentivector, as defined by titering the vector on a cell line such as HeLa or 293. Unit doses range from $10^3$, $10^4$, $10^5$, $10^6$, $10^7$, $10^8$, $10^9$, $10^{10}$, $10^{11}$, $10^{12}$, $10^{13}$ T.U. and higher.

## K. EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Materials and Methodology Employed in Examples 1 Through 3

Cell Lines and Culture Conditions

293T, F208 and Hela P4 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Cell were cultured in incubators at 37° C. in a humidified 5% $CO_2$ atmosphere.

Plasmids Construction

All plasmid modifications were done according to standard procedures (Sambrook et al. 1989).

Plasmid pHIV(BRU) contains the full-length proviral genome of HIV-1 strain BRU. Plasmid pcPPT-D contains the full-length genome of HIV-1 but the cPPT/cTS sequence element is mutated as described in SEQ ID NO:1 and SEQ ID NO:4.

Plasmid pHIV(BRU)ΔE was constructed by replacing the SalI-BamHI fragment with the corresponding fragment from pR9ΔE. Plasmid pcPPT-D ΔE was constructed similarly.

Plalsmid pHIV(BRU) Δ 1470 was constructed by replacing the SalI-BamHI fragment with the corresponding fragment from pCMVA R8.91. Plasmid pcPPT-D Δ 1470 was constructed similarly.

Vector Preparation

Stocks of vector were prepared as previously described (Zufferey, et al. 1997, Zufferey, et al. 2000, incorporated herein by reference). Three or four plasmids were transiently cotransfected into 293T cells to generate second and third generation lentiviral vector, respectively. Vector preparation and cell transduction were done in a BL2 laboratory. Reverse transcriptase activity was measured in each vector stock using the method described in Klages et al. (2000), incorporated herein by reference. Differences in reverse transcriptase activity, usually less than 15%, were corrected by dilution of the stocks with high activity.

Virus Stocks Preparation

Stocks of virus were prepared by transfecting the different proviral plasmids into 293T cells. For pseudotyping experiments, envelope defective proviral plasmid were cotransfected with the pMD.G plasmid encoding the VSV G protein. Reverse transcriptase activity was measured in each virus stock using the method described in Klages et al. (2000). Differences in reverse transcriptase activity, usually less than 15%, were corrected by dilution of the stocks with high activity.

Vector Titration

Vectors were titrated on 293T and F208 cells. Target cell ($5\times10^4$ cells/well) were plated in each well of a 6-well tissue culture plate and incubated 24 hour in 1 ml DMEM. Vector stocks (100 μl) or 4 serial 1:10 dilutions was added to 1 ml of fresh DMEM and incubated for 2 more days. Polybrene was omitted.

Flow Cytometry Analysis

Cells were analyzed as described (Arrighi et al., 1999), on a FACScalibur (Becton-Dickinson) with slight modifications. FL-1 was used for GFP, FL-2 for autofluorescence. Cells were fixed with 2% paraformaldehyde for 30 minutes, and resuspended into PBS prior to analysis. Data were analyzed using WINMDI™ software written by J. Trotter at Scripps Institute (La Jolla, Calif.) and CellQuest software (Becton-Dickinson).

Virus Titration on HeLa P4 Cells

HeLa P4 cells express human CD4 and contain a reporter transgene made of HIV-1 LTR fused to the *E. coli* LacZ gene. Upon HIV-1 infection and genome integration, the HIV-1 Tat protein is produced. This protein trans-activates the HIV-1 LTR promoter activity resulting in high expression level of β-galactosidase encoded by the Lac Z gene. β-galactosidase activity is detected by an histochemical staining HIV-1 infected cells acquire a blue color. The histochemical detection of β-galactosidase is described in Zufferey, et al. (2000).

HeLa P4 cells ($5\times10^4$ cells/well) were plated in each well of a 6-well tissue culture plate and cultured in 1 ml DMEM for 24 hour before being infected. For infection, 1 ml of cPPT deficient vector stock was used whereas 1 ml and dilutions corresponding to 10, 5 and 1 μl were used for the wild-type HIV-1. The number of blue foci were counted using an inverted light microscope in wells containing less than 100 infection events.

### Example 1

### Modification of a Lentiviral Packaging Plasmid Central Polypurine Tract (cPPT/cTS)

Modifications to the sequence of the cPPT/cTS region may be made so that nuclear import is severely hindered without

interfering with the activity of the pol gene of which the cPPT/cTS region is a part (Zennou, et al., 2000). Incorporated into a packaging plasmid, such sequences will be represented in any RCRs that may arise during the production or use of lentiviral vectors and so will effectively inhibit the nuclear import of these undesired RCRs. The packaging plasmid pMDLD incorporates modifications to the cPPT/cTS region that effectively inhibit nuclear import of lentiviral genomes (FIG. **1**).

Plasmid pMDLD is derived from pMDLg/pRRE, which has been described fully elsewhere (Dull, et al., 1998, incorporated herein by reference). Briefly, pMDLg/pRRE is a CMV-driven expression plasmid that contains only the gag and pol coding sequences from HIV-1. Additionally, a 374-bp RRE-containing sequence from HIV-1 (HXB2) is present immediately downstream of the pol coding sequences. An inactive cPPT/cTS region was substituted for that of pMDLD by replacing the wild-type AflII-BspEI fragment (positions 5296 to 5760 of the plasmid) with the corresponding AflII-BspEI fragment of SEQ ID NO:1. The resulting sequence differs in the cPPT/cTS region, positions 5432 through 5452 as indicated in FIG. **1** and described in SEQ ID NO:4.

The mutations which inactivate the cPPT/cTS region do not impact on the function of the integrase protein. To test whether the novel packaging systems have conserved their ability to produce HIV-1 vectors, vector production by systems with an active or an inactive cPPT/cTS regions were compared. Vectors encoding the Green Fluorescent Protein (GFP) were generated by transient co-transfection of 293T cells with four plasmids according to previously published protocols (Zufferey, et al. 2000). The transfer vector used in these experiments was the transfer vector plasmid pRRL-CMV GFP SIN. The envelope plasmid used was pMD.G, which encodes the vesicular stomatitis virus G protein. The pRSVrev plasmid encoding the HIV-1 Rev protein was also used.

The resulting vector stocks were assayed for reverse transcriptase activity to eliminate any difference which could result from variability in transfection efficiency. Stocks with matched reverse transcriptase activity were titrated on 293T cells and F208 cells. For titration, $10^5$ cells were plated in each well of 6-well plates and cultured in 1 ml of medium. 24 hours after plating, cells were transduced with 500 microliters of vector stock or of serial dilutions of vector stocks.

The percentage of GFP-expressing cells was determined 48 hours later by Fluorescence Activated Cell Sorting (FACS). With both cell lines, we found that vector titers were independent from the functionality of the cPPT/cTS sequence element in the packaging plasmids (FIG. **2**). Thus, the cPPT/cTS sequence element can be inactivated in plasmids for the packaging of HIV-1 based vectors without any decrease in vector titers produced. The increase in biosafety conferred by the modification can be effectively incorporated into useful methods for the production of lentiviral vectors.

## Example 2

### Insertion of a Stuffer Sequence into the Packaging Plasmid Enhances Biosafety

The biosafety of modifications to the sequence of the cPPT/cTS region is enhanced when the overall length of any resultant RCR genome is sufficiently large. Such an increase in size is obtained by inserting a stuffer sequence into the packaging plasmid pMDLD described above.

To test whether the infectivity of the HIV-1 virus in the absence of an active cPPT/cTS sequence element depends on viral genome size, we have generated HIV-1 proviral genomes of decreasing size by removing sequence stretches encoding the envelope protein or accessory proteins. The missing genetic information was provided in trans to complement the defective viruses. The relative infectivity of HIV 1 viruses with different genome sizes was assayed on P4 cells.

The decreasing genome size did not affect the infectivity of the viruses containing a cPPT/cTS sequence element. In contrast, the infectivity of the mutated viruses increased when the genome size was reduced. For each genome size, we performed pairwise comparisons of viruses with or without an active cPPT/cTS sequence element. For viruses of wild-type length, we found that the HIV-1 virus with an inactive cPPT/cTS sequence element is 200 times less infectious than its wild-type counterpart. For viruses shorter by 1470 nucleotides, the virus with the inactive cPPT/cTS sequence element is only 70 times less infectious than its wild-type counterpart.

The inhibitory effect on viral replication due to the absence of the cPPT/cTS function increases with the viral genome size. Consequently, the size of the packaging plasmids for the production of lentiviral vectors may be increased in order to maximize the safety improvement obtained by the inactivation of the cPPT/cTS sequence element. The size of the packaging plasmids can be increased by inserting DNA at different positions. The highest safety is obtained by inserting DNA between the end of gag/pol gene and the RRE sequence element because DNA inserted at this position is most likely included in the genome of a putative recombinant virus.

## Example 3

### Creation of Replication Inhibiting Mutant 5' LTR Poly(A) Hairpins

Some mutations in the 5' R-U5 region of the Long Terminal Repeat (LTR) have profound inhibitory effects on virus replication (Das, et al., 1997). Two mutated 5' LTR poly(A) hairpin sequences (mutA and mutC, corresponding to SEQ ID NO: 5 and SEQ ID NO: 6, respectively) were selected from a panel of altered poly(A) hairpin sequences as disclosed by Das, et al., (1997), (see FIG. **8**). These two mutants were chosen because they have the strongest inhibitory effect on virus replication. Previous partial characterization of these mutations suggested that the mutations might affect a step of viral replication that is not required for the vector function.

To test the effects of these mutant sequences, mutations A and C in the R region of the 5' LTR were introduced into the plasmid pHR'CMV GFP SIN (Zufferey, et al., 1998) so as to replace the wild-type sequence. Vector was produced using the wild-type or mutated transfer vector plasmids in combination with pCMV ΔR8.91 as packaging plasmid and pMD.G as envelope plasmid expressing the VSV G protein. The packaging plasmid pCMV ΔR8.91 is an HIV-derived packaging construct, which encodes the HIV-1 Gag and Pol precursors, as well as the regulatory proteins Tat and Rev (Zufferey et al., 1997). Vectors stocks were produced by transient transfection of 293T cells according to published protocol (Zufferey, et al., 1997), matched for reverse transcriptase activity and titrated on 293T and F208 cells as described.

Substantially identical titers for wild-type and mutC vectors were displayed. For the mutA vector, titers were apparently reduced by a factor of 10. Since the mutation A abolishes the function of the viral polyA addition signal, the comparison was repeated using wild-type and mutated versions of the pHR'pA-GFP-I-AC plasmid in which the GFP transgene has it own polyA signal independent from the LTR.

In this setting the titers of all three vector stocks were identical. These mutations in the R region of LTR can severely impair the replication of the HIV-1 virus without affecting the production and the transduction efficiency of an HIV-1 derived vector.

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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<210> SEQ ID NO 16
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What is claimed is:

1. A method for transducing a target animal cell comprising contacting the target animal cell with a recombinant lentiviral vector under conditions to effect the transduction of the target animal cell by the recombinant lentiviral vector, wherein the recombinant lentiviral vector is produced by a method comprising:

(I) (a) transfecting a host cell with: (i) a packaging plasmid comprising a stuffer sequence and a cPPT/cTS region that has reduced replication activity relative to wild-type cPPT/cTS replication activity, wherein the packaging plasmid comprises a pol gene and an RRE, and the stuffer sequence is positioned between the pol gene and the RRE of the packaging plasmid; (ii) an expression plasmid, which carries an env gene; and (iii) a lentiviral transfer vector comprising a therapeutic transgene; to yield a producer cell; (b) culturing the producer cell in a medium; and separating the producer cell from the medium to recover the recombinant lentiviral vector from the medium;

(II) (a) transfecting a host cell with: (i) a packaging plasmid, wherein the packaging plasmid comprises a stuffer sequence and a cPPT/cTS region that has reduced replication activity relative to wild-type cPPT/cTS replication activity, wherein the stuffer sequence is of sufficient length to effectively provide a lentivirus having at least the size of a wild type lentiviral genome; (ii) an expression plasmid, which carries an env gene; and (iii) a lentiviral transfer vector comprising an expression cassette comprising a therapeutic transgene positioned under the control of a promoter that is active to promote detectable transcription of the transgene in a cell, a 3' LTR and a 5' LTR, wherein the 5' LTR comprises a poly(A) hairpin sequence that inhibits viral replication to yield a producer cell; (b) culturing the producer cell in a medium; and (c) separating the producer cell from the medium to recover the recombinant lentiviral vector from the medium; or

(III) (a) transfecting a host cell with: (i) a packaging plasmid, wherein the packaging plasmid comprises an RRE, a cPPT/cTS region that has reduced replication activity relative to wild-type cPPT/cTS replication activity and a stuffer sequence, wherein the stuffer sequence is of sufficient length to effectively provide a lentivirus having at least the size of a wild-type lentiviral genome; (ii) an expression plasmid, which carries an env gene; and (iii) a lentiviral transfer vector comprising an expression cassette comprising a transgene positioned under the control of a promoter that is active to promote detectable transcription of the transgene in a cell, a 3' LTR and a 5' LTR, wherein the 5' LTR comprises a poly(A) hairpin sequence that inhibits viral replication; to yield a producer cell; (b) culturing the producer cell in a medium; and (c) separating the producer cell from the medium to recover the recombinant lentiviral vector from the medium.

2. The method of claim 1, wherein the recombinant lentiviral vector is produced by a method comprising:

(I) (a) transfecting a host cell with: (i) a packaging plasmid comprising a stuffer sequence and a cPPT/cTS region that has reduced replication activity relative to wild-type cPPT/cTS replication activity, wherein the packaging plasmid comprises a pol gene and an RRE, and the stuffer sequence is positioned between the pol gene and the RRE of the packaging plasmid; (ii) an expression plasmid, which carries an env gene; and (iii) a lentiviral transfer vector comprising a therapeutic transgene; to yield a producer cell; (b) culturing the producer cell in a medium; and (c) separating the producer cell from the medium to recover the recombinant lentiviral vector from the medium.

3. The method of claim 1, wherein the recombinant lentiviral vector is produced by a method comprising:

(II) (a) transfecting a host cell with: (i) a packaging plasmid, wherein the packaging plasmid comprises a stuffer sequence and a cPPT/cTS region that has reduced replication activity relative to wild-type cPPT/cTS replication activity, wherein the stuffer sequence is of sufficient length to effectively provide a lentivirus having at least the size of a wild type lentiviral genome; (ii) an expression plasmid, which carries an env gene; and (iii) a lentiviral transfer vector comprising an expression cassette comprising a therapeutic transgene positioned under the control of a promoter that is active to promote detectable transcription of the transgene in a cell, a 3' LTR and a 5' LTR, wherein the 5' LTR comprises a poly(A) hairpin sequence that inhibits viral replication to yield a producer cell; (b) culturing the producer cell in a medium; and (c) separating the producer cell from the medium to recover the recombinant lentiviral vector from the medium.

4. The method of claim 1, wherein the recombinant lentiviral vector is produced by a method comprising:

(III) (a) transfecting a host cell with: (i) a packaging plasmid, wherein the packaging plasmid comprises an RRE, a cPPT/cTS region that has reduced replication activity relative to wild-type cPPT/cTS replication activity and a stuffer sequence, wherein the stuffer sequence is of sufficient length to effectively provide a lentivirus having at least the size of a wild-type lentiviral genome; (ii) an expression plasmid, which carries an env gene; and (iii) a lentiviral transfer vector comprising an expression cassette comprising a transgene positioned under the control of a promoter that is active to promote detectable transcription of the transgene in a cell, a 3' LTR and a 5' LTR, wherein the 5' LTR comprises a poly(A) hairpin sequence that inhibits viral replication; to yield a producer cell; (b) culturing the producer cell in a medium; and (c) separating the producer cell from the medium to recover the recombinant lentiviral vector from the medium.

5. The method of claim 1, wherein the target animal cell is a human cell.

6. The method of claim 1, wherein the target animal cell is a hematopoietic cell.

7. The method of claim 1, wherein the hematopoietic cell is obtained from bone marrow, peripheral blood or umbilical cord blood.

8. The method of claim 1, wherein the target animal cell is a CD34+ cell.

9. The method of claim 1, wherein the target animal cell is a hematopoietic stem cell.

10. The method of claim 1, wherein the target animal cell is a hematopoietic progenitor cell.

11. The method of claim 1, wherein the target animal cell is a hematopoietic progenitor cell.

12. The method of claim 1, wherein the target animal cell is a peripheral blood B lymphocyte cell.

13. The method of claim 1, wherein the target animal cell is a peripheral blood T lymphocyte cell.

14. The method of claim 1, wherein the target animal cell is a CD4+ T lymphocyte cell.

15. The method of claim 1, wherein the target animal cell is a peripheral blood mononuclear cell.

16. The method of claim 1, wherein the target animal cell is a fetal cord blood cell, a fibroblast cell, a brain cell, a lung cell, a liver cell, a muscle cell, a pancreatic cell, an endothelial cell, a cardiac cell, a skin cell, a bone marrow stromal cell, and an eye cells, a pancreatic ductal cell, a neural precursor, or a mesodermal stem cell.

17. The method of claim 1, wherein the cell is transduced in vivo.

18. The method of claim 1, wherein the target animal cell is transduced in vitro.

19. The method of claim 1, wherein the target animal cell is transduced ex vivo.

20. The method of claim 1, wherein the transduced target animal cell is administered to an animal subject.

21. The method of claim 1, wherein the animal subject is a mammal.

22. The method of claim 1, wherein the animal subject is a primate.

23. The method of claim 1, wherein the animal subject is a human.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

# CERTIFICATE OF CORRECTION



| | |
|---|---|
| PATENT NO. | : 9,260,725 B2 |
| APPLICATION NO. | : 14/525520 |
| DATED | : February 16, 2016 |
| INVENTOR(S) | : Didier Trono and Romain N. Zufferey |

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the claims:

In claim 1, column 53, line 18, between "and" and "separating", insert --(c)--.

Signed and Sealed this
Fifth Day of July, 2016

Michelle K. Lee

Michelle K. Lee
*Director of the United States Patent and Trademark Office*